Cytotechnology **40:** 31–38, 2002.

Murine Peyer's patch dendritic cells prime naïve CD4$^+$ T cells to produce interferon-γ

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Received 26 November 2001; accepted in revised form 21 January 2002

Key words: dendritic cells, IFN-γ, IL-12, mucosal immunity, Peyer's patch

Abstract

We investigated the role of Peyer's patch (PP) dendritic cells (DCs) in the production of interferon (IFN)-γ from naïve CD4$^+$ T cells of T cell receptor transgenic mice. PP DCs were found to prime naïve CD4$^+$ T cells for the production of higher levels of IFN-γ, when compared to spleen (SP) DCs. However, a similar level of interleukin-12 (IL-12) production was observed for PP and SP DCs stimulated via the CD40 molecule. In addition, PP DCs expressed slightly higher levels of B7.2 (CD86) compared to SP DCs. This data demonstrates that PP DCs have a distinct function in the induction of IFN-γs and suggests that PP DCs may enhance IFN-γ production via another cytokine or costimulatory molecule, in addition to IL-12.

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FAE, follicle-associated epithelium; FITC, fluorescein isothiocyanate; OVA, ovalbumin; PP, Peyer's patch; SED, subepithelial dome; SP, spleen; TCR, T-cell receptor; IFN, interferon; IL, interleukin.

Introduction

Production and secretion of antibodies of the IgA isotype characterize immune responses at mucosal surfaces, which represent the first line of defense against colonization by many pathogens (Mestecky and McGhee, 1987). Most IgA antibody-containing cells originate in mucosa-associated lymphoid tissue, such as Peyer's patches (PP) in the intestine, where they encounter antigen. One of the unique features of PP is their capacity to induce Th cell production of Th2 cytokines (interleukin-4 (IL-4), IL-5, IL-10) (Daynes et al., 1990) and Th3 (TGF-β) cytokines (Santos et al., 1994), which are important for IgA production. However, despite this capacity to generate Th2/Th3 responses in PP, distinct Th1 responses can be induced in the mucosa, particularly after intestinal infection with pathogenic microorganisms. For instance, interferon (IFN)-γ secretion by PP T cells has been observed after gastrointestinal infection with *Salmonella typhimurium* (Everest et al., 1997; George, 1996; Hess et al., 1996; Karem et al., 1996) and *Toxoplasma gondii* (Liesenfeld et al., 1997). In addition, high dose antigen feeding results in a transient Th1 response before T cell clonal deletion (Marth et al., 1996).

Although it is known that antigens and microorganisms from the intestinal lumen are transported into PP by M cells present in the follicle-associated epithelium (FAE), the details of immune regulation in the PP are just beginning to be understood. The qualitative and quantitative nature of an immune response depends on the type of antigen-presenting cell (APC) that processes and presents antigen to the T cells. Dendritic cells (DCs) are the most potent antigen-presenting cells known for the induction of primary T cell responses (Banchereau et al., 1998). In addition, previous studies have suggested that DCs are a major antigen-presenting cell population in the subepithelial dome (SED) of the PP, the region just beneath the FAE (Mayrhofer et al., 1983; Spencer et al., 1986; Witmer-Pack et al., 1993). Freshly isolated PP DCs

are functionally distinct from splenic DCs with regard to their capacity to induce T helper cell differentiation *in vitro* (Iwasaki and Kelsall, 1999). PP DCs were shown to prime naïve $CD4^+$ antigen-specific T cells for the secretion of IL-10, IL-4 and IFN-γ, whereas splenic DCs primed predominantly for the secretion of IFN-γ. These studies have provided important information concerning the possible role of DCs in antigen presentation in PP.

In the present study, we also address the issue of whether freshly isolated DCs from PP differ from DCs from spleen (SP) in their ability to induce T cell differentiation and cytokine production. Since DCs are the most potent APCs to prime naïve T cells, we examined the ability of PP DCs to induce cytokine secretion from naïve T cells. PP DCs were found to prime T cells for higher production of IFN-γ compared to SP DCs. PP DCs were found to produce similar levels of IL-12 p40, and express slightly higher levels of B7.2 (CD86), when compared to SP DCs. These results suggest that PP DCs enhance IFN-γ production via a cytokine other than IL-12, or via alternative costimulatory molecules.

Materials and methods

Mice

Female BALB/c mice were purchased from Clea Japan Inc. (Tokyo, Japan) and used at 6–7 weeks of age. Chicken ovalbumin (OVA)-specific T-cell receptor (TCR) transgenic mice (OVA 23–3 (Sato et al., 1994)) were maintained by backcrossing to BALB/c mice. Almost all $CD4^+$ T cells from these mice expressed the $\alpha\beta$-TCR that recognized OVA peptide (amino acids 323–339) restricted to I-A^d. Female OVA 23–3 mice, were used at 18–22 weeks of age.

Culture media

Cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 3 mM L-glutamine, and 50 μM β-mercaptoethanol.

Antibodies

Hamster anti-mouse CD11c (N418) and anti-CD62L (MEL-14) were purified from their respective hybridomas, and biotinylated using a standard protocol. Rat anti-mouse B220 (CD45R; RA3-6B2), anti-CD4-fluorescein isothiocyanate (FITC) (H129.19), anti-mouse CD16/CD32 (2.4G2), hamster anti-mouse CD40 (HM40-3), anti-hamster IgM (G188-2), hamster anti-mouse CD80 (16-10A1), and rat anti-mouse CD86 (GL1) were purchased from BD PharMingen (San Diego, CA).

Preparation of DCs

DCs were prepared from PP and SP of naïve 6-week old BALB/c mice in parallel. PP and SP were digested with collagenase Type I (1 mg ml^{-1}; Sigma, St. Louis, MO), and a single cell suspension was prepared. The cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotech, Auburn, CA) and selected using MACS separation columns. Cells selected on the basis of CD11c expression were stained with biotinylated hamster anti-mouse CD11c antibody, streptavidin-PE and FITC-labeled anti-B220 antibody. $CD11c^+$ $B220^-$ cells were purified by flow cytometric sorting performed using a FACS Vantage (Becton Dickinson Immunocytometries, Mountain View, CA). Sorted DC populations were routinely 97–100% positive for the cell surface marker of interest.

Stimulation of DCs in vitro

FACS-purified DC populations were preincubated with hamster anti-mouse CD40 (50 μg ml^{-1}), and then incubated with anti-hamster IgM (5 μg ml^{-1}) to crosslink the anti-CD40. Supernatants were harvested at 48 h and IL-12 levels were measured by enzyme-linked immunosorbent assay (ELISA).

Stimulation of TCR transgenic T cells by DCs

Naïve SP $CD4^+$/$CD62L^{high}$ T cells from OVA-specific TCR transgenic mice (OVA23-3) were prepared using a preparative magnetic cell sorter (MACS, Miltenyi Biotech, Auburn, CA).

Primary stimulation cultures were established by coincubation of MACS-purified naïve OVA TCR $CD4^+$T cells from SP (1×10^5 per well) with sorted $CD11c^+$ $B220^-$ DCs from SP or PP (1×10^4 per well). Cells were incubated with OVA (1 mg ml^{-1}) in 96-well plates for 48 h. Supernatants were then collected, and the cytokine levels were measured by ELISA. For inducing the development of effector T cells from naïve T cells, primary stimulation of cultures with OVA (100 μg ml^{-1}) was performed for 3 days. The

cells were then transferred to 24-well plates and allowed to expand for 4 days in fresh medium, without additional antigens. The T cells were then washed, and 1×10^5 cells were coincubated with 9×10^5 X-irradiated spleen cells plus OVA (100 μg ml^{-1} or 1 mg ml^{-1}) in 96-well plates. After 48 h of incubation, the supernatants were harvested and cytokine levels were measured by ELISA.

Measurement of cytokine secretion

Cytokine production in the culture supernatants was assayed by specific sandwich ELISA. ELISAMaxisorp immunoplates (NUNC, Roskilde, Denmark) were coated with rat anti-mouse IL-2 (JES6-1A12, BD PharMingen), rat anti-mouse IL-4 (11B11, BD PharMingen), rat anti-mouse IL-5 (TRFK5, BD PharMingen), rat anti-mouse IL-6 (MP5-20F3, BD PharMingen), rat anti-mouse IL-12 (C15.6, BD PharMingen) or rat anti-mouse IFN-γ (R4-6A2, BD PharMingen). After washing and blocking of the plates, the samples and standards were added. Bound cytokine was detected using biotin-labeled monoclonal anti-mouse IL-2 (JES6-5H4, BD PharMingen), rat anti-mouse IL-4 (BVD6-24G2, BD PharMingen), rat anti-mouse IL-5 (TRFK4, BD PharMingen), rat anti-mouse IL-6 (MP5-32C11, BD PharMingen), rat anti-mouse IL-12 (C17.8, BD PharMingen) or rat anti-mouse IFN-γ (XMG1.2, BD PharMingen), respectively. This was followed by incubation with alkaline phosphatase (AP)-labeled streptavidin (Zymed). The plates were then washed and the substrate (*p*-nitrophenylphosphate) was added. Absorbance was determined at a wavelength of 405 nm. IL-10 was measured using the OptEIA™ set (BD PharMingen).

Surface phenotype analysis of DC populations from PP and SP

CD11c$^+$/B220$^-$ cells were concentrated using magnetic beads. The cells were stained with biotinylated hamster anti-mouse CD80 or biotinylated rat anti-mouse CD86 antibody, and then stained with streptavidin-PE, biotinylated hamster anti-mouse CD11c antibody, streptavidin-CyChrome, and FITC-labeled anti-B220 antibody. CD11c$^+$/B220$^-$ cells were analyzed for expression of CD80 and CD86 molecules by flow cytometry.

Results and discussion

We investigated the induction of cytokine production of naïve T cells from the T cell receptor transgenic mice, OVA 23–3, using PP or SP DCs as antigen-presenting cells. Almost all of the CD4$^+$ T cells from these mice express T cell receptors, that specifically recognize the OVA 323–339 peptide in the context of I-A^d on a BALB/c background. Using this transgenic mouse model, it is possible to detect the cytokine response of naïve T cells after antigen presentation. In this experiment, cells from PP and SP of BALB/c mice were sorted by flow cytometry to obtain a 97–100% pure CD11c$^+$/B220$^-$ dendritic cell population (Figure 1). Sorted DCs and purified naïve T cells were cultured with OVA, and the cytokine levels present in the culture supernatant were measured by ELISA. As shown in Figure 2, we observed that primary stimulation of naïve T cells with antigen presented by PP DCs, but not by SP DCs, induced the production of the Th1 cytokine IFN-γ by these cells. Antigen presentation by both PP DCs and SP DCs induced T cell production of IL-2. IL-4, IL-5 and IL-10 were not detected using the ELISA.

Since PP DCs induced secretion of IFN-γ from naïve T cells, we next examined whether PP DCs induced differentiation of IFN-γ secreting Th1 cells. Naïve T cells from OVA TCR transgenic mice were primed using the sorted BALB/c DCs from PP or SP. Seven days later, the T cells were collected, washed, and restimulated with OVA and APC. After 48 h, culture supernatants were collected and cytokine levels were measured by ELISA. Secondary stimulation of PP DC-primed T cells with OVA resulted in the secretion of IL-2, as well as high levels of IFN-γ, indicating that PP DCs induced Th1 differentiation (Figure 3). The level of IFN-γ production by T cells primed by the PP DCs was higher than that of T cells primed using the SP DCs. In addition, high levels of IL-4, IL-5, IL-6 and IL-10 were also secreted from PP DC-primed T cells, suggesting that Th2 cells were also induced. These results demonstrated that PP DCs induced development of both Th1 and Th2 cells, and are similar to the results reported by Iwasaki and Kelsall (1999). These results imply that under some conditions, PP DCs can induce stronger Th1 differentiation than SP DCs.

One possible mechanism underlying the distinct cytokine production patterns induced by stimulation of T cells with PP and SP DCs, in particular the higher levels of IFN-γ production induced by PP DCs in

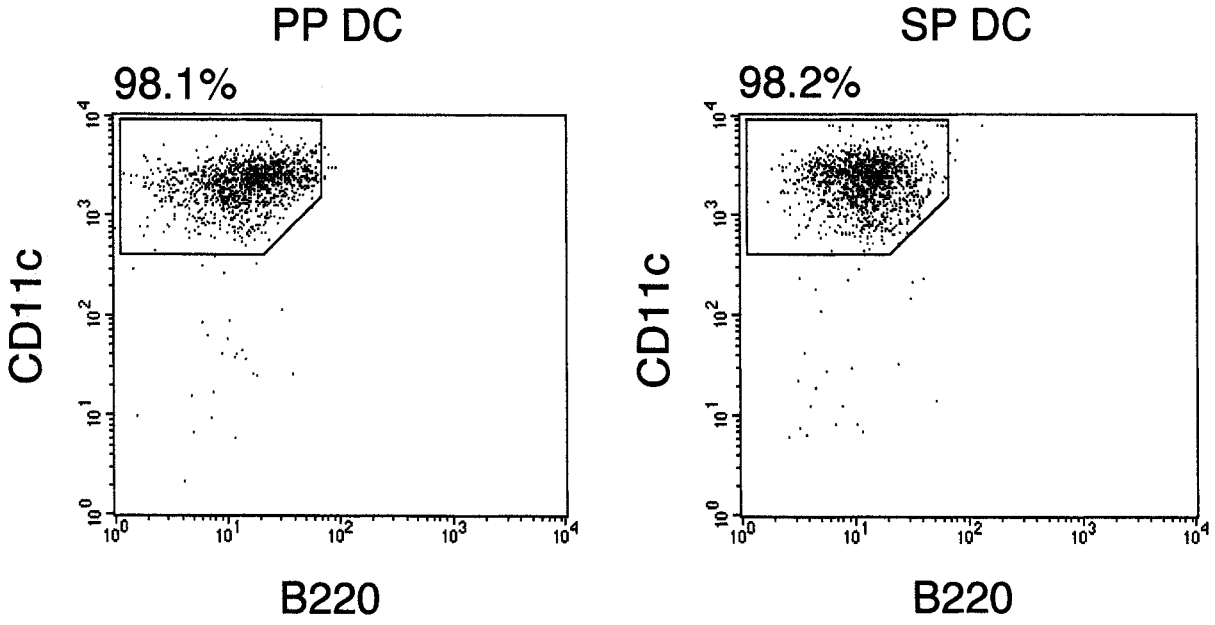

Figure 1. Sorted DC population from PP and SP. Cells from PP or SP were isolated using magnetic beads as described in the Materials and Methods Section, and were dual stained with the biotinylated CD11c antibody, streptavidin-PE and the FITC- conjugated anti-B220 antibody. Subsequently the $CD11c^{+}/B220^{-}$ cells were purified by flow cytometric sorting indicated by the enclosed polygon.

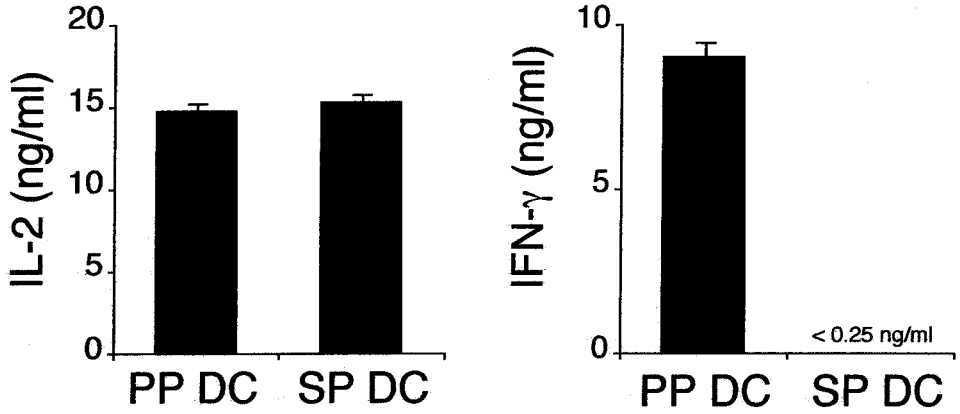

Figure 2. Cytokine production by naïve $CD4^{+}$ T cells induced by antigen presentation of sorted DC from PP and SP. MACS-purified naïve $CD4^{+}/CD62L^{high}$ T cells from SP of OVA TCR transgenic mice (1×10^{5} per well) were coincubated with DCs (1×10^{4} per well) from PP or SP for 48 h in the presence of OVA (1 mg ml^{-1}). Supernatants were harvested and the cytokine levels were measured by ELISA. Data is representative of two separate experiments that produced similar results.

primary culture, may be that these DCs secrete discrete sets of cytokines upon activation by T cells. To address this possibility, cytokine production by purified DCs in the absence of T cells was assessed. DCs are known to secrete IL-12, which is an important cytokine for induction of IFN-γ secretion by T cells. There are several potent stimulation pathways that induce IL-12 production by DCs. One of these pathways is a T cell-dependent interaction via the CD40-CD40 ligand and/or MHC class II-TCR (Cella et al., 1996; Kato et al., 1997; Koch et al., 1996). IL-12 production by DCs through the CD40-CD40L interaction is thought to be important for Th1 induction (Macatonia et al., 1995; Maldonado-Lopez et al., 1999). Thus, we examined cytokine secretion by cross-linking cell surface CD40 molecules. As shown in Figure 4, stimulation with anti-CD40 antibody induced IL-12 secretion from both PP and SP DCs. Although SP DCs stim-

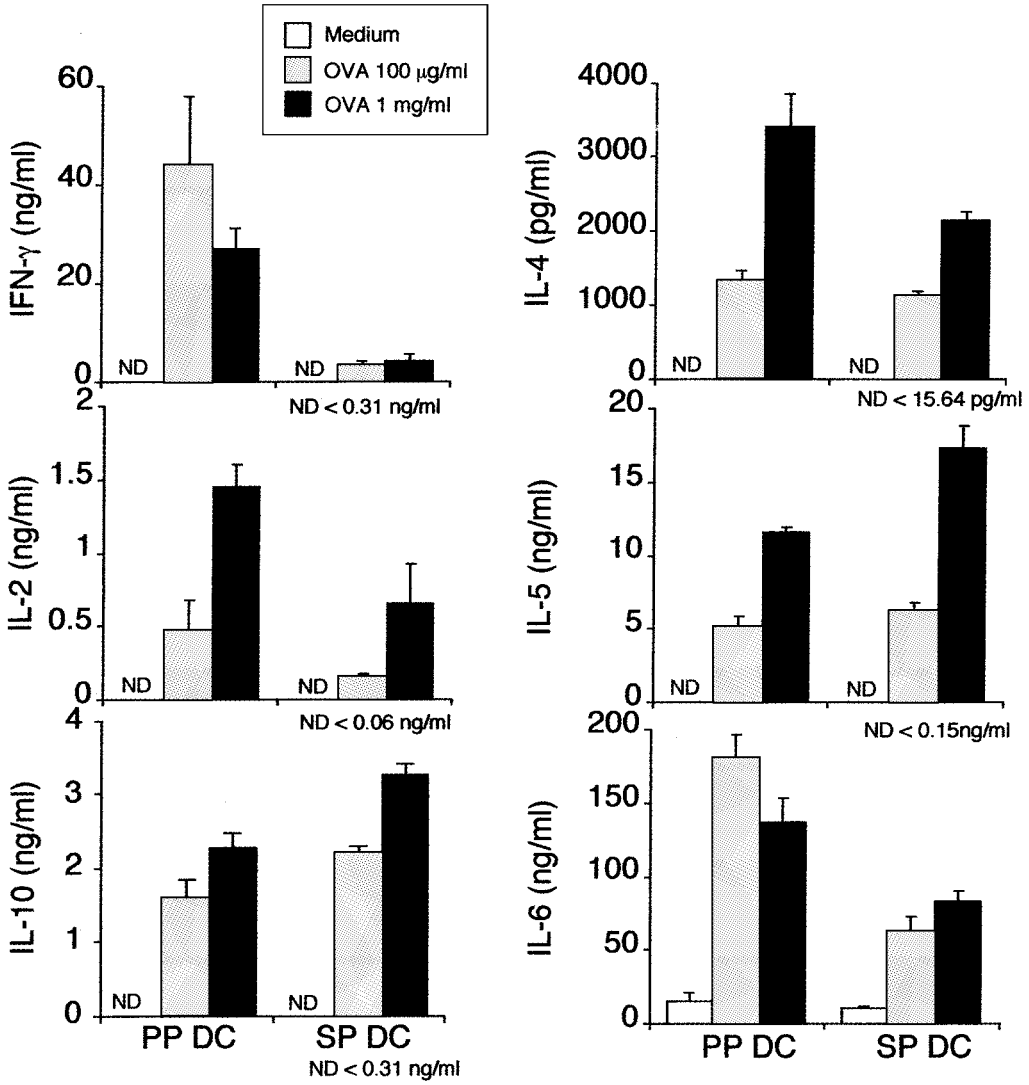

Figure 3. Cytokine production by TCR transgenic T cells after secondary stimulation. MACS-purified naïve $CD4^+/CD62L^{high}$ T cells from SP of OVA TCR transgenic mice (1×10^5 per well) were coincubated with DCs (1×10^4 per well) sorted from PP or SP in the presence of OVA (100 μg ml^{-1}). After 3 d, cells were transferred to 24-well plates and allowed to expand for 4 d in fresh medium without additional antigens. T cells were then washed and 1×10^5 cells were coincubated with 9×10^5 irradiated BALB/c spleen cells plus OVA (100 μg ml^{-1} or 1 mg ml^{-1}) in 96-well plates. After 48 h, supernatants were harvested and the cytokine levels were measured by ELISA. Data is representative of three separate experiments that produced similar results.

ulated with anti-CD40 mAb secreted IL-12 as well as PP DCs (Figure 4), SP DCs induced lower IFN-γ secretion compared to PP DCs (Figure 2). Only the IL-12 heterodimer (IL-12 p70), composed of two subunits p35 and p40, has biological activity. Figure 4 shows the sum of p70 and p40 homodimer. Secretion of IL-12 p70 could not be detected by ELISA for DCs stimulated with the anti-CD40 mAb. In addition, p35 mRNA was not detected using RT-PCR. As such, it appears unlikely that the difference in IFN-γ induction is due to a difference in the levels of IL-12 secretion.

Ohteki et al. (1999) found that DCs, as well as T and NK cells, were capable of producing IFN-γ when cultured in the presence of IL-12. In our experiment, however, we were unable to detect IFN-γ by ELISA from cultures of PP and SP DCs stimulated via the CD40 molecule. We presume from this that all of the IFN-γ was produced by the T cells, as indicated in Figure 2.

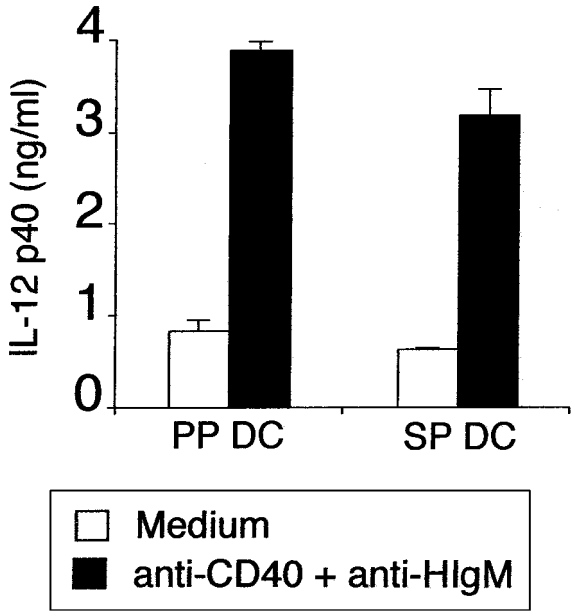

Figure 4. IL-12 production by sorted PP DCs after stimulation. Sorted DCs (5×10^4 per well) from BALB/c mice were incubated in the presence of stimulators. Cells were preincubated with hamster anti-mouse CD40 mAb (50 μg ml^{-1}), and then incubated with anti-hamster IgM (5 μg ml^{-1}) to crosslink the anti-CD40 mAb. Supernatants were harvested and the cytokine levels were measured by ELISA at 48 h. Data is representative of five separate experiments, each of which produced similar results.

Cytokines other than IL-12, or alternative costimulatory molecules, may account for the higher level of IFN-γ secretion in response to antigen presentation by PP DCs, as compared to SP DCs. Iwasaki et al. (1999) reported that IL-10 secreted by DCs inhibited induction of IFN-γ secretion. In our experiment, we were unable to detect IL-10 by ELISA in the supernatant from cultures of PP or SP DCs stimulated via the CD40 molecule. However, it may be possible that a small amount of IL-10 suppressed the production of IFN-γ. Recently, a novel p19 protein was found to engage IL-12 p40 to form the complex known as IL-23 (Oppmann et al., 2000). IL-23 is naturally expressed by activated mouse and human dendritic cells and has biological activities that are similar to IL-12. Although IL-23 induced secretion of IFN-γ from T cells, mouse IL-23 was found to stimulate activated memory T cells, but not naïve T cells. Since we used naïve T cells, there was little possibility that higher IFN-γ production induced by PP DCs was due to IL-23.

T cell-dependent immune responses initiated by DCs depend on their expression of specific costimulatory molecules. Among the most important costimulatory signals are those delivered by the B7 family, which is currently composed of five members with demonstrated immunologic activity. A selectivity of B7.1 (CD80) for promoting Th1 responses, and B7.2 (CD86) for promoting Th2 responses, has been described in mice (Kuchroo et al., 1995; Lenschow et al., 1995; Miller et al., 1995). Although PP DCs induced naïve T cells to secrete higher IFN-γ when compared to SP DCs, isolated PP DCs were found to express an equivalent level of B7.1 (CD80), and slightly higher levels of B7.2 (CD86), than SP DCs (Figure 5). Many reports have suggested different functions for B7.1 and B7.2 in Th1/Th2 differentiation, however, the molecular basis for these signaling effects remain to be clarified. Alternatively, it is possible that PP DCs enhance IFN-γ production via other costimulatory molecules. A new B7 family member, B7-DC, whose expression is highly restricted to DCs, was recently identified (Tseng et al., 2001). B7-DC costimulates T cell proliferation more efficiently than B7.1, and induces a distinct pattern of lymphokine secretion. B7-DC strongly enhances IFN-γ secretion, but not IL-4 or IL-10 production, from isolated naïve T cells. B7-DC is an important DC mediator of Th1 responses in addition to IL-12. There is a possibility that PP DCs express higher levels of B7-DC than SP DCs, with IFN-γ production induced by PP DCs promoted by this costimulatory molefule.

It is well established that Th1 type responses are important in both innate and acquired immunity. Several studies have addressed the role of IFN-γ in host defense against microorganisms. Upon Ag capture, or microbial infection, DCs produce various cytokines such as IL-12 that act on T lymphocytes to induce IFN-γ production (Banchereau et al., 1998). Our results suggest that PP DCs may play an important role in host defense at the intestinal mucosa by inducing IFN-γ secretion.

Recently, Iwasaki and Kelsall (2000) identified three distinct subsets of DCs in murine PP by immunohistochemical analysis. One population of DCs, which are CD11b$^+$/CD8α^- and are of the myeloid lineage, reside in the SED region. Another DC subset that are CD11b$^-$/CD8α^+ (lymphoid DCs), reside in the T cell-rich interfollicular region (IFR). DCs that lack expression of CD11b or CD8α (double negative) are present in both the SED and IFR. Efforts to investigate the mechanism of IFN-γ induction by PP DCs are currently underway by analyzing the phenotype of PP DCs.

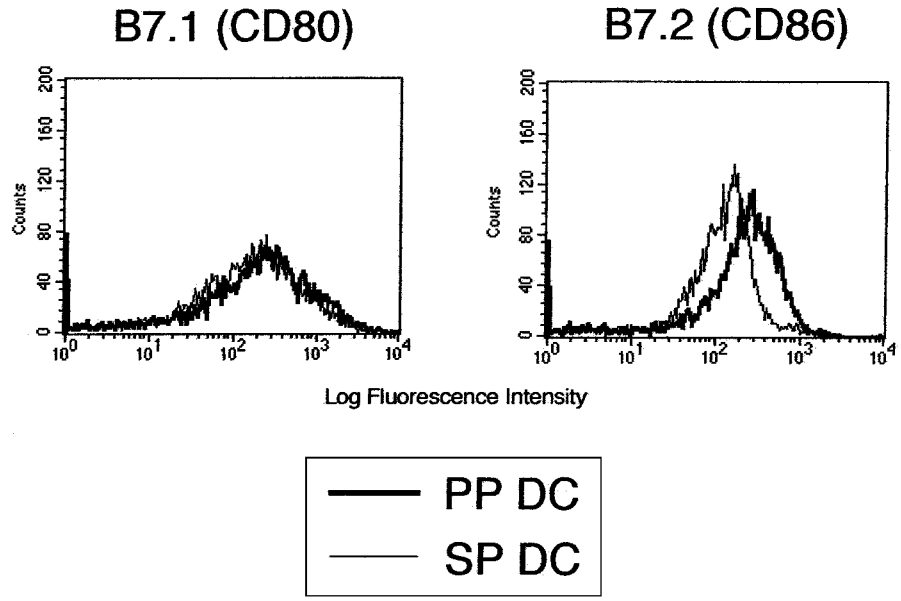

Figure 5. Surface phenotype analysis of DC populations ($CD11c^+/B220^-$) from PP or SP. $CD11c^+/B220^-$ DCs from PP or SP were analyzed for expression of cell surface CD80 and CD86 molecules. The results are shown as histograms with fluorescence intensity on the x-axis, and cell number on the y-axis. The thin lines represent staining of SP DCs, and the thick lines staining of PP DCs. The data depicted here represents five independent experiments, each of which produced similar results.

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