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J Immunol 2003; 171:3684-3690; ;
doi: 10.4049/jimmunol.171.7.3684
http://www.jimmunol.org/content/171/7/3684

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

CD11b$^+$ Peyer's Patch Dendritic Cells Secrete IL-6 and Induce IgA Secretion from Naive B Cells[1]

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Peyer's patch (PP) dendritic cells (DCs) have been shown to exhibit a distinct capacity to induce cytokine secretion from CD4$^+$ T cells compared with DCs in other lymphoid organs such as the spleen (SP). In this study, we investigated whether PP DCs are functionally different from DCs in the SP in their ability to induce Ab production from B cells. Compared with SP DCs, freshly isolated PP DCs induced higher levels of IgA secretion from naive B cells in DC-T cell-B cell coculture system in vitro. The IgA production induced by PP DCs was attenuated by neutralization of IL-6. In addition, the induction of IgA secretion by SP DCs, but not PP DCs, was further enhanced by the addition of exogenous IL-6. Finally, we demonstrated that only PP CD11b$^+$ DC subset secreted higher levels of IL-6 compared with other DC subsets in the PP and all SP DC populations, and that PP CD11b$^+$ DC induced naive B cells to produce higher levels of IgA compared with SP CD11b$^+$ DC. These results suggest a unique role of PP CD11b$^+$ DCs in enhancing IgA production from B cells via secretion of IL-6. ***The Journal of Immunology,*** **2003, 171: 3684–3690.**

Although it is known that Ags and microorganisms from the intestinal lumen are transported into the Peyer's patch (PP)[5] by M cells present in the follicle-associated epithelium, the details of immune regulation within the PP are just beginning to be understood. PP represents the primary site for Ag processing and presentation in the intestine. Previous studies have suggested that dendritic cells (DCs) are the major APC population in the subepithelial dome (SED) of the PP, the region just beneath the follicle-associated epithelium (1–3). In addition, isolated DCs from the PP have been shown to stimulate T cells in MLR (4, 5) and to support IgA B cell differentiation (6–9). Moreover, it was reported that freshly isolated PP DCs are functionally distinct from DCs from the spleen (SP) with regard to their capacity to induce Th cell differentiation in vitro (5); PP DCs were shown to prime naive CD4$^+$ Ag-specific T cells to secrete IL-10, IL-4, and IFN-γ, whereas SP DCs predominantly primed CD4$^+$ T cells to secrete IFN-γ. In addition, only PP DCs were shown to produce significant levels of IL-10 after stimulation in vitro with recombinant CD40 ligand (5). These prior studies have provided important information concerning the particular role of DCs in Ag presentation in the PP.

More recently, Iwasaki and Kelsall (10, 11) identified three distinct subsets of DCs in murine PP by immunohistochemical analysis. One population of DCs, CD11b$^+$/CD8α^- DCs, reside in SED region, whereas another subset, CD11b$^-$/CD8α^+ DCs, reside in the T cell-rich interfollicular region (IFR), and DCs that lack expression of CD11b or CD8α (double negative) are present in both the SED and IFR. All DC subpopulations maintained their surface phenotype upon maturation in vitro. These data suggested that these DC subsets are derived from different lineages. CD11b$^+$ DCs from PP produce high levels of IL-10 upon in vitro stimulation. In contrast, CD8α^+ and double negative DCs, but not CD11b$^+$ DCs, produce IL-12p70 following microbial stimulation. Moreover, CD11b$^+$ DCs from PP were found to induce T cell differentiation to secrete high levels of IL-4 and IL-10, when compared with those from nonmucosal sites, whereas CD8α^+ and double negative DCs from all tissues induced IFN-γ production. This suggested that CD11b$^+$ DCs from PP have unique immune inductive capacities.

Immune responses at the mucosal surfaces including the intestinal epithelium are characterized by the protection mediated by the secreted IgA. The PP has been considered to be the major inductive site for IgA. The following studies demonstrated that PP DCs play an important role in the induction of IgA production. Induction of significant polyclonal IgA secretion both by PP and SP B cells was shown in coculture of B cells with DC-T cell mixtures derived from murine PP but not SP (7). Nontransformed pre-B cells were induced to differentiate in vitro to secrete IgA with only DCs and Ag-stimulated T lymphocytes (8). This study showed that PP DCs induced high levels of IgA in combination with T cells derived from either the SP or the PP, and that both the isotype of Ab secreted and the extent of pre-B cell differentiation were determined by the lymphoid tissue source of DC, not of T cells. The importance and sufficiency of PP DCs in IgA induction from B cells were demonstrated in a study by George et al. (9), in which PP DCs, but not Th cells, were able to promote IgA secretion from B cells in a microculture. Although these data suggested the importance of PP DCs in the generation of IgA responses in the

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Received for publication December 4, 2002. Accepted for publication July 22, 2003.

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[1] This work was supported in part by a grant-in-aid for Creative Scientific Research (13GS0015), a grant-in-aid for Scientific Research, and a grant-in-aid for Young Scientists, the Japan Society for the Promotion of Science, and by a program for the Promotion of Basic Research Activities for Innovative Biosciences.

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[5] Abbreviations used in this paper: PP, Peyer's patch; DC, dendritic cell; SED, subepithelial dome; IFR, interfollicular region; SP, spleen; Tg, transgenic.

0022-1767/03/$02.00

intestine, the mechanisms of induction of IgA production by PP DCs still remain unclear.

Previous studies have shown that cytokines, such as TGF-β (12–14), IL-5 (15–20), and IL-6 (19, 21, 22) are important factors for the development of IgA-producing B cells. PP is not the only inductive site for IgA-secreting plasma cells in the gut. Several studies have identified that up to 40% of IgA cells in the intestinal lamina propria arise from a pool of B-1 precursors derived from the peritoneal cavity (23), which are distinct from the conventional PP precursors by their anatomical origin, function, and expression of the CD5 surface marker (24, 25). $CD5^+$ peritoneal cavity B cells are able to produce IgA in vitro in the absence of IL-6 (26). Therefore, more than one pathway exists for IgA production, and that by $CD5^+$ B-1 cells is IL-6 independent. On the other hand, the potential role of IL-6 in IgA production by B-2 cells, which are the predominant B cells in the PP, has been supported by analysis of IL-6-deficient mice (22).

In this report, we demonstrate that PP DCs, particularly the $CD11b^+$ DCs found in the SED region, can produce higher levels of IL-6 compared with SP DCs. Moreover, we demonstrate that upon in vitro culture, PP DCs, especially the $CD11b^+$ subset, have a particular capacity to induce IgA production compared with SP DCs. This effect of the PP DCs is largely diminished upon addition of IL-6 neutralizing Ab in the DC-T-B coculture. These data suggest that PP DCs have a unique ability to secrete IL-6 and contribute to the establishment of IgA secretion in the intestinal mucosa.

Materials and Methods

Mice

Female BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and used at 6–7 wk of age. OVA-specific TCR transgenic (Tg) mice (OVA 23–3 (27) and DO11.10 TCR Tg mice (28) were maintained by backcrossing to BALB/c mice. Female Tg mice were used at 8–22 wk of age.

Culture medium

Cells were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 3 mM L-glutamine, and 50 μM 2-ME

RT-PCR

Total RNA was isolated from sorted DC populations by RNeasy (Qiagen, Hilden, Germany). Single-stranded cDNA was synthesized using SuperScript preamplification system (Life Technologies, Gaithersburg, MD). PCR was conducted for 40 cycles using primer pairs for IL-6, forward, 5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′; reverse, 5′-TCTGACCACAGTGAGGAATGTCCAC-3′ and GAPDH, forward, 5′-TGAACGGGAAGCTCACTGG-3′; reverse, 5′-TCCACCACCCTGTTGCTGTA-3′ and for fluorophore-labeled hybridization probes for IL-6, forward, 5′-LC-AACCTAGTGCGTTATGCCTAAGC-3′; reverse, 5′-AAGTCACTTTGAGATCTACTCGGC-FITC-3′ and GAPDH, forward, 5′-LC-TTCAACAGCAACTCCCACTCTTCCACC-3′; reverse, 5′-CTGAGGACCAGGTTGTTGTCTCCTGCGA-FITC-3′ with the LightCycler (Roche Diagnostics, Rotkreuz, Switzerland).

Antibodies

Surface phenotype of DCs was analyzed with purified mAb as follows. Hamster anti-mouse CD11c was purified from hybridoma, N418 obtained from the American Type Culture Collection (HB224). Rat anti-mouse CD8α (53-6.7) was purchased from BD PharMingen (San Diego, CA). Purified monoclonal rat anti-mouse CD11b (M1/70) was purchased from Caltag Laboratories (Burlingame, CA). Before staining, Fc receptors (FcγRIII/II) were blocked using anti-mouse CD16/CD32 (2.4G2). Naive T cells from OVA TCR Tg mice were stained with anti-mouse CD4-FITC (H129.19) and anti-mouse CD62L-biotin (MEL-14) and Streptavidin-PE. For isolation of naive B cells, anti-mouse IgD (Southern Biotechnology Associates, Birmingham, AL) was used. For stimulation to DC, hamster anti-mouse CD40 (HM40-3) and anti-hamster IgM (G188-2) were purchased from BD PharMingen.

Preparation of whole DCs and subpopulation of DCs

DCs were prepared from PP and SP of naive 6-wk-old mice in parallel. PP and SP were digested with collagenase type I (1 mg/ml; Sigma-Aldrich, St. Louis, MO), and a single cell suspension was prepared. The cells were incubated with anti-mouse CD11c-coated magnetic beads (Miltenyi Biotec, Auburn, CA) and selected on MACS separation columns (Miltenyi Biotec). Cells selected on the basis of CD11c expression were stained with biotinylated hamster anti-mouse CD11c Ab, streptavidin-PE and FITC-labeled anti-B220 Ab. $CD11c^+/B220^-$ cells were purified by flow cytometric sorting performed using a FACSVantage (BD Immunocytometry Systems, Mountain View, CA). For isolation of DC subpopulations, cells selected on the basis of CD11c expression by MACS were stained with biotinylated hamster anti-mouse CD11c Ab and streptavidin-Red670 (Life Technologies) and FITC-labeled anti-B220 Ab and either PE-labeled anti-CD11b or PE-labeled CD8α, and then $CD11b^+/CD11c^+/B220^-$ and $CD8\alpha^+/CD11c^+/B220^-$ DCs were sorted by using FACSVantage. Sorted DC populations were routinely 97–100% positive for the surface marker of interest.

Stimulation of DCs in vitro

FACS-purified DC populations were preincubated with hamster anti-mouse CD40 (50 μg/ml) and then incubated with anti-hamster IgM (5 μg/ml) to cross-link anti-CD40. Alternatively, DCs were incubated with PMA (50 ng/ml) and Ca^{2+} ionophore (A23187, 250 ng/ml), or with LPS (20 μg/ml). Supernatants were harvested at 48 h, and IL-6 levels were measured by ELISA.

Stimulation of TCR Tg T Cells by DCs or DC populations

Naive SP $CD4^+$ $CD62L^{high}$ T cells from OVA-specific TCR Tg mice (OVA 23–3) were prepared using MACS. Isolated naive T cells were routinely 95–97% positive for $CD4^+$ $CD62L^{high}$.

Primary stimulation cultures were established by coincubation of MACS-purified naive OVA TCR Tg $CD4^+$ T cells from SP (1×10^5 per well) and sorted $CD11c^+/B220^-$, $CD11b^+/CD11c^+/B220^-$, or $CD8\alpha^+/CD11c^+/B220^-$ DCs from SP or PP (1×10^4 per well). Cells were incubated with OVA (1 mg/ml) in 96-well plates for 48 h. Supernatants were collected, and then IL-2, IL-4, IL-5, IL-6, IL-10, and IFN-γ levels were measured by ELISA.

Induction of Ig production by DCs

Naive SP $CD4^+$ $CD62L^{high}$ T cells from splenocytes of TCR Tg mice (DO11.10 TCR Tg mice) were prepared using MACS. Naive B cells were isolated from SP of BALB/c mice. SP were digested with collagenase, and surface (s)IgD^+ B lymphocytes were separated using MACS. Isolated B cells were routinely 95–98% positive for the surface marker of $sIgD^+/B220^+$. $sIgD^+$ B lymphocytes (2.5×10^5 per well) and MACS-purified OVA TCR Tg naive T cells from SP (2×10^5 per well) and sorted $CD11c^+/B220^-$ DCs or $CD11b^+/CD11c^+/B220^-$ or $CD11b^-/CD11c^+/B220^-$ DC subsets from SP or PP (1×10^4 per well) were cultured in the presence or absence of OVA (100 μg/ml) on 96-well plates for a week. Recombinant IL-6 (10 ng/ml, BD PharMingen), neutralizing Ab against IL-6 (10 μg/ml, BD PharMingen), or rat IgG1 as isotype control (10 μg/ml, BD PharMingen) was added to some cultures. Supernatants were collected, and IgA, IgM, IgG1, and IgG2a levels were measured by ELISA.

Measurement of cytokine and Ig secretion

Cytokine and Ig productions in the culture supernatants were assayed by specific sandwich ELISA. A number of 96-well 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 IFN-γ (R4-6A2, BD PharMingen), goat anti-mouse IgA (Zymed Laboratories, South San Francisco, CA), goat anti-mouse IgM (Organon Teknika, Durham NC) and goat anti-mouse IgG (Sigma-Aldrich). After washing and blocking the plates, samples and standards ware 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) or rat anti-mouse IFN-γ (XMG1.2, BD PharMingen), respectively. This was followed by incubation with alkaline phosphatase-labeled streptavidin (Zymed). For Ig quantification, alkaline phosphatase-conjugated monoclonal anti-mouse IgA, IgM, IgG1, and IgG2a (Zymed) were used. The plates were then washed and the substrate (disodium *p*-nitrophenylphosphate) was added. Absorbance was determined at a wavelength of 405 nm. IL-10 was measured using the OptEIA set (BD PharMingen).

Results

Induction of Ig production by DCs from PP and SP

In initial experiments, to address whether PP DCs have a particular capacity to induce IgA production, we compared Ig production levels in an in vitro culture system in which DCs from PP or SP were used as the APCs. Freshly isolated PP or SP DCs were purified on the basis of surface marker ($CD11c^+/B220^-$) by flow cytometric sorting. Naive $sIgD^+$ B lymphocytes isolated from SP of BALB/c mice, naive T cells from SP of OVA TCR Tg mice, and sorted $CD11c^+/B220^-$ DCs from either PP or SP were cocultured in the presence of OVA. Fig. 1 is representative data that depict the levels of IgA secreted in these DC-T-B coculture systems. Consistently, higher IgA production was observed when PP DCs were used as the APCs compared with those wells containing SP DCs ($p < 0.01$). In some experiments, we observed a tendency for PP DCs to induce higher levels of IgM production compared with SP DCs as shown in Fig. 1 (not statistically significant); however, in other experiments, there was no difference between cultures containing PP DCs or SP DCs (data not shown). IgG1 or IgG2a levels did not differ significantly between PP DCs and SP DC cultures either. To examine the importance of DC in directing IgA secretion from B cells, we have analyzed IgA production levels in either CD3-stimulated T cell-B cell culture or Ag-stimulated T cell-B cell culture. Only background levels of IgA production was observed from B cells cultured with T cells in the presence of OVA Ag (no DC in Fig. 1), or with anti-CD3 activated T cells (data not shown). These results showed that IgA production observed in DC-T-B culture was not merely the result of B cell help provided by T cells activated by the DCs, but revealed a specific function of the PP DCs.

To investigate the role of IL-6, a critical factor for the development of IgA-producing B cells (19, 21, 22), we measured Ig production levels in culture in the presence of either anti-mouse IL-6 mAb or exogenous rIL-6. We found that IgA production induced by PP DCs was substantially decreased in the presence of anti-mouse IL-6 mAb (Fig. 1). In the presence of additional rIL-6, IgA levels were found to be enhanced in cultures containing SP DCs as the APCs. Although this was not statistically significant, the addition of rIL-6 consistently enhanced IgA production in SP DC-T-B cell culture in multiple experiments. In contrast, there was no further enhancement in IgA secretion by additional IL-6 provided in PP DC-T-B coculture. Neutralizing IL-6 decreased IgG1 and IgG2a, although this was not statistically significant except for IgG2a induced by SP DCs, whereas addition of rIL-6 resulted in the enhancement of IgG1 and IgG2a induced by SP DCs (IgG1, $p < 0.05$; IgG2a, $p < 0.01$), and tendency of higher IgG1 and IgG2a levels when PP DCs were used as APCs. However, these effects were observed similarly in cultures containing PP or SP DC as the APCs. Thus, these data suggested that PP DCs have a capacity to induce IgA production and that IL-6 is important for the DC-induced IgA secretion.

Higher levels of IL-6 are induced in cultures with PP DCs as the APCs

Because it was likely that PP DCs regulate IgA responses through stimulation of T cells, we investigated the induction of cytokine

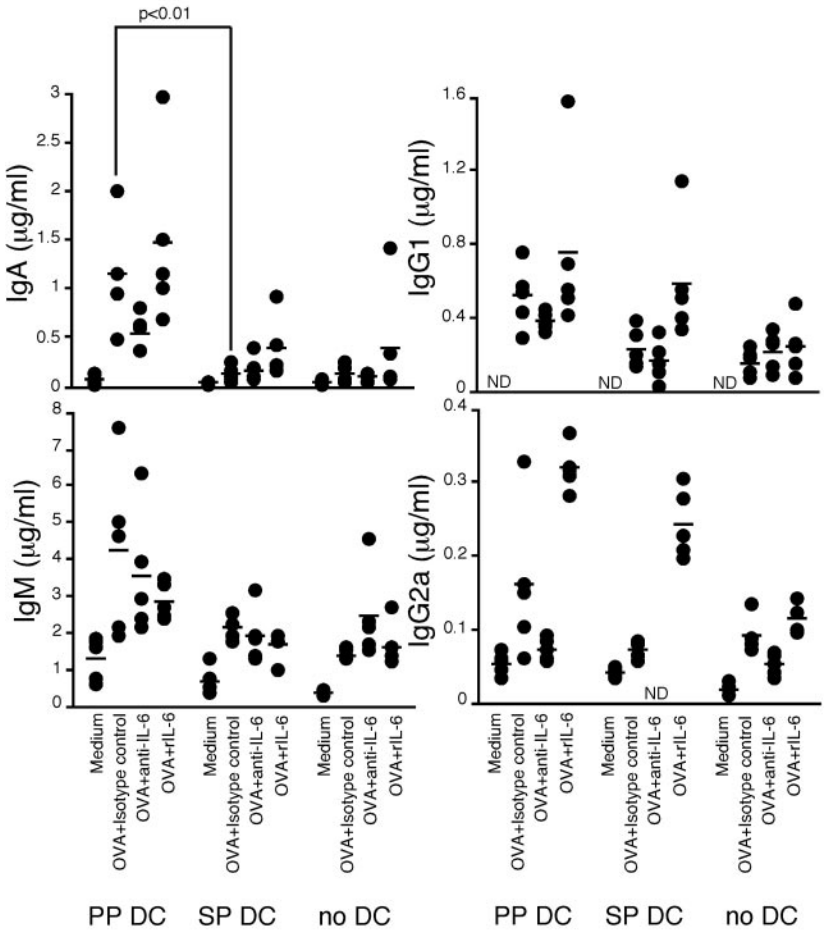

FIGURE 1. Ig production induced by freshly isolated PP or SP. MACS-purified naive $CD4^+/CD62L^{high}$ T cells from SP of OVA TCR Tg mice (2×10^5 per well) and MACS-purified naive IgD^+ B cells from SP of BALB/c mice (2.5×10^5 per well) were coincubated with DCs (1×10^4 per well) from PP or SP for 7 days in the presence or absence of OVA (100 μg/ml). Either rIL-6 (10 ng/ml), neutralizing Ab against IL-6 (10 μg/ml), or isotype control (10 μg/ml) was added to some cultures as indicated. Supernatants were harvested and Ig levels were measured by ELISA. Results for individual culture wells are indicated and the crossbars represent the mean value. Detection limits of IgG1 and IgG2a were 0.010 μg/ml. ND, not detected. Data are representative of five separate experiments producing similar results.

production by PP DCs or SP DCs as the APCs using naive $CD4^+$ T cells from OVA TCR Tg mice. As shown in Fig. 2, we observed that higher levels of IFN-γ and IL-6 were produced in T cell stimulation cultures with PP DCs as APCs compared with cultures with SP DCs. IL-4, IL-5, and IL-10 were not detected by ELISA.

The IL-6 detected in the DC-T coculture could be secreted by either DCs and/or T cells. We thus investigated whether purified PP DCs produced IL-6. Analysis of IL-6 secretion from sorted DCs following stimulation with either anti-CD40 mAb and anti-hamster IgM (for cross-linking), PMA and Ca^{2+} ionophore (A23187), or LPS revealed that DCs stimulated with all stimulators secreted significant levels of IL-6, and that PP DCs secreted higher levels of IL-6 compared with SP DCs (Fig. 3). We also investigated the expression of IL-6 mRNA by sorted DCs after stimulation with the same stimuli. As shown in Table I, PP DCs in response to all stimuli expressed higher levels of IL-6 RNA compared with SP DCs. Further freshly isolated PP DCs expressed higher levels of IL-6 mRNA than SP DCs even in the absence of in vitro stimulation (Table I). Thus, PP DCs express higher levels of IL-6 mRNA and secrete significantly higher amounts of IL-6, and Ag presentation by PP DCs to $CD4^+$ T cells induce higher levels of IL-6 production compared with splenic DCs.

$CD11b^+$ DCs from PP can produce higher levels of IL-6

We investigated which subpopulation of the PP DCs produce IL-6. Freshly sorted DC subpopulations from PP and SP, namely $CD11b^+/CD11c^+/B220^-$ and $CD8\alpha^+/CD11c^+/B220^-$ populations (Fig. 4, *A* and *B*), were stimulated with either anti-CD40 and anti-hamster IgM, PMA and Ca^{2+} ionophore (A23187), or LPS. As shown in Fig. 4*C*, $CD11b^+$ DCs from the PP secreted higher levels of IL-6 compared with $CD8\alpha^+$ DCs from the PP and SP DCs. To confirm this finding, we also compared the production by sorted $CD11b^+$ and $CD11b^-$ DCs (Fig. 4*D*), or $CD8\alpha^+$ and $CD8\alpha^-$ DCs from the PP (Fig. 4*E*). In response to CD40 cross-linking, $CD11b^+$ DCs from the PP secreted higher levels of IL-6 compared with $CD11b^-$ DCs, and $CD8\alpha^-$ DCs from the PP secreted higher levels of IL-6 compared with $CD8\alpha^+$ DCs. Therefore, $CD11b^+/CD8\alpha^-$ DCs from the PP can secrete higher levels of IL-6 compared with the splenic counterpart. Neither $CD8\alpha^+/CD11b^-$ DCs nor $CD8\alpha^-/CD11b^-$ double negative DCs from either PP or SP secreted appreciable levels of IL-6.

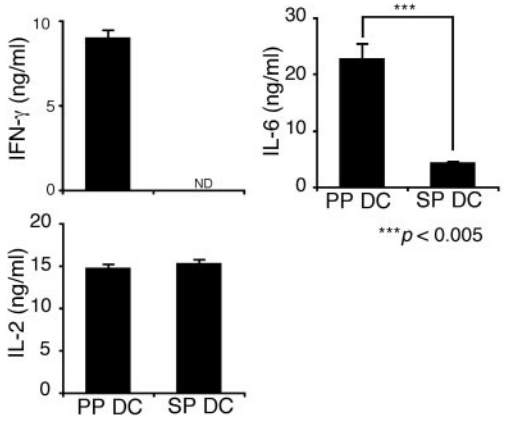

FIGURE 2. Cytokine productions induced by the Ag presentation of sorted DC from PP and SP during primary culture. MACS-purified naive $CD4^+/CD62L^{high}$ T cells from SP of OVA TCR Tg mice (1×10^5 per well) were coincubated with DCs (1×10^4 per well) from PP or SP for 48 h in the presence of OVA (1 mg/ml). Supernatants were harvested and cytokine levels were measured by ELISA. ND, not detected. Detection limits were 0.25 ng/ml. Data are representative of two separate experiments producing similar results.

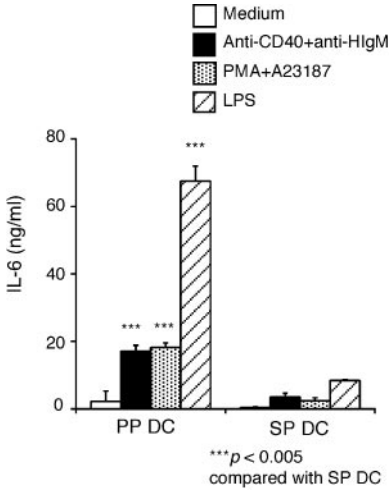

FIGURE 3. IL-6 production by sorted PP DCs after stimulation. Sorted DCs (5×10^4 per well) from BALB/c mice were incubated in the presence of stimulators. Cells were preincubated with hamster anti-mouse CD40 mAb (50 μg/ml), and then incubated with anti-hamster IgM (5 μg/ml) to cross-link the anti-CD40 mAb, incubated with PMA (50 ng/ml)+A23187 (250 ng/ml), or incubated with LPS (2 μg/ml). Supernatants were harvested and the cytokine levels were measured by ELISA at 48 h. Data are representative of five separate experiments producing similar results.

We investigated which subset of PP DCs induce IL-6 production in the in vitro culture of DCs and naive $CD4^+$ T cells. As shown in Fig. 5, PP $CD11b^+$ DCs were found to induce higher levels of IL-6 production in the presence of specific Ag compared with other DC populations ($p < 0.005$, when compared with other DC population). Therefore, $CD11b^+$ PP DCs have a unique ability to secrete IL-6 and also to induce IL-6 secretion in DC-$CD4^+$ T cell cultures in an Ag-specific manner.

Induction of Ig production by DC subsets from PP and SP

Finally, we investigated whether $CD11b^+$ DCs isolated from PP induce higher levels of IgA production in DC-T-B cell coculture. As shown in Fig. 6, PP $CD11b^+$ DCs were found to induce higher levels of IgA production in the presence of specific Ag compared with other DC populations, in particular, there was a statistically significant difference when compared with SP $CD11b^+$ DCs ($p < 0.05$).

Table I. *Expression of IL-6 RNA by freshly isolated or stimulated DCs*[a]

	Experiment 1		Experiment 2	
	PP DC	SP DC	PP DC	SP DC
In vitro-stimulated DCs[b]				
Medium (control)	2.1	2.5	3.5	6.5
Anti-CD40 + anti-HIgM	9.4	4.9	6.6	2.8
PMA + A23187	8.4	2.7	10.8	4.1
Freshly isolated DCs[c]	10.7	1.8	2.1	0.6

[a] cDNA samples prepared from freshly isolated or cultured DCs were analyzed by quantitative RT-PCR for expression of IL-6 and a control marker, GAPDH. Expression levels of IL-6 by PP DCs and SP DCs are presented as the ratios of IL-6 total RNA levels to GAPDH total RNA levels derived from the same cDNA sample.

[b] Freshly isolated DCs were cultured for 20 h (Experiment 1: 5×10^4 per well; Experiment 2: 1×10^4 per well) in the presence or absence of stimulators. Cells were stimulated as described in Fig. 3.

[c] cDNA samples prepared from freshly isolated PP and SP DCs from BALB/c mice.

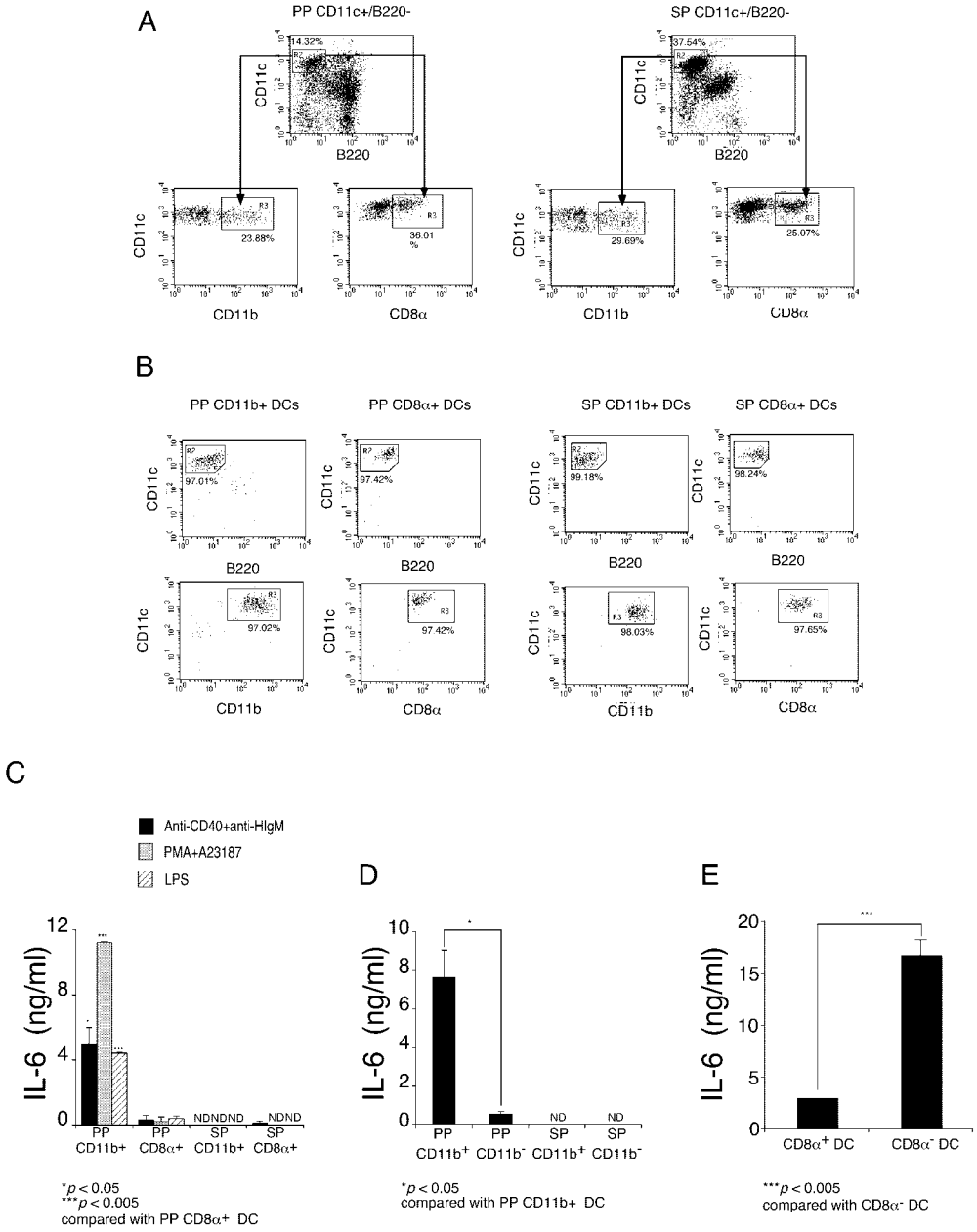

FIGURE 4. The FACS profiles defining the sorting gates used to isolate CD8α^+ or CD11b$^+$ DCs. *A*, Sorting gates used to isolate DC subsets. *B*, Purified DC subsets. IL-6 productions by sorted PP DC subpopulations after stimulation. Sorted DC subpopulations (2×10^4 per well) from BALB/c mice were incubated in the presence of stimulators. *C*, CD8α^+ or CD11b$^+$ DCs. *D*, CD11b$^+$ or CD11b$^-$ DCs. *E*, CD8α^+ or CD8α^- PP DCs. Cells were stimulated as described in Fig. 3. Supernatants were harvested and IL-6 levels were measured by ELISA at 48 h. ND, not detected. Detection limits were 0.312 ng/ml (*C*) and 0.156 ng/ml (*D*). Data are representative of two separate experiments producing similar results.

Discussion

To study the mechanism by which mucosal DCs induce IgA production in B cells, we examined the cytokine secretion and Ag presentation to CD4$^+$ T cells by PP DCs and compared them to SP DCs. We demonstrated that Ag presentation by PP DCs promoted higher amounts of IL-6 production compared with SP DCs, in cultures containing naive T cells and specific Ag. Further, analysis of IL-6 production by purified DCs in the absence of T cells revealed that PP DCs secreted higher levels of IL-6 compared with SP DCs in response to cross-linking of CD40 molecules (Fig. 3, Table I).

PP DCs were found to secrete high levels of IL-6 not only in response to anti-CD40 mAb but also to LPS. These results suggest that PP DCs secrete high levels of IL-6 during interaction with T cells, and in response to microbial components. Indeed, freshly sorted PP DCs expressed an IL-6 mRNA level higher than did SP DCs without stimulation (Table I). Because an enormous amount of food and microbial Ags are continuously being processed within the PP, PP DCs may be in a constant state of activation. This is supported by our observation that freshly isolated PP DCs possessed high levels of IL-6 mRNA not seen in SP DCs.

IL-6 markedly and selectively enhances IgA production in vitro by isotype-committed B cells (21, 29). It has been reported that mice with targeted disruption of the gene encoding IL-6 mount very poor IgA responses in the intestine and lungs, but that this defect can be overcome following vector-directed IL-6 gene therapy (22). These mice are also markedly deficient in IgA-producing plasma cells in the mucosa. We hypothesized that PP DCs enhance

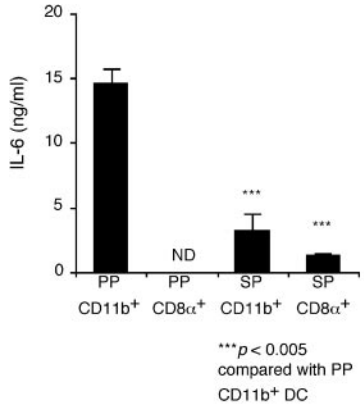

FIGURE 5. IL-6 production induced by the Ag presentation of sorted DC populations from PP and SP during primary culture. MACS-purified naive $CD4^+/CD62L^{high}$ T cells from SP of OVA TCR Tg mice (1×10^5 per well) were coincubated with DCs (1×10^4 per well) from PP or SP for 48 h in the presence of OVA (1 mg/ml). Supernatants were harvested and IL-6 levels was measured by ELISA. ND, not detected. Detection limit was 0.62 ng/ml. Data are representative of two separate experiments producing similar results.

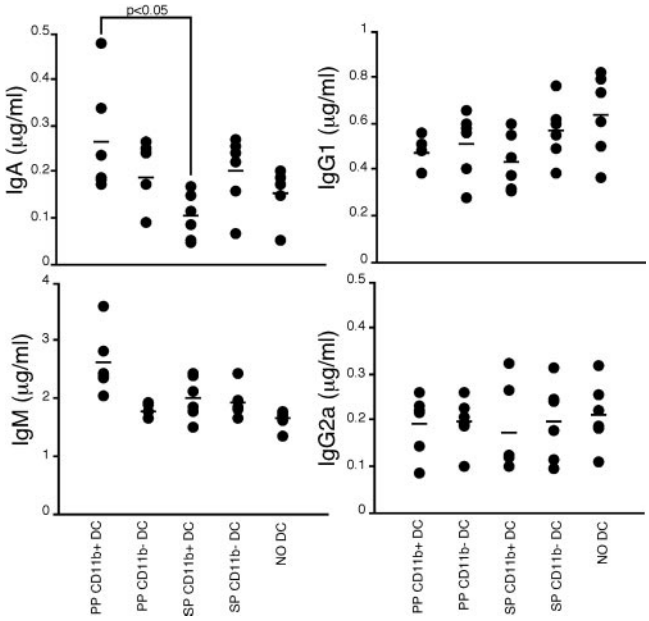

FIGURE 6. Ig production induced by freshly isolated DC populations from PP and SP. MACS-purified naive $CD4^+/CD62L^{high}$ T cells from SP of OVA TCR Tg mice (2×10^5 per well) and MACS-purified naive IgD^+ B cells from SP of BALB/c mice (2.5×10^5 per well) were coincubated with DCs (1×10^4 per well) from PP or SP for 7 days in the presence of OVA (100 μg/ml). Supernatants were harvested and Ig levels were measured by ELISA. Results for individual culture wells are indicated and the crossbars represent the mean value. Data are representative of two separate experiments producing similar results.

IgA production by inducing high levels of IL-6 secretion from $CD4^+$ T cells. To address the issue, we investigated Ig production induced by PP or SP DCs in the DC-T-B coculture. We observed that PP DCs, especially the $CD11b^+$ subset, induced higher levels of IgA production from naive B cells in the in vitro culture compared with SP DCs (Figs. 1 and 6). We extend previous findings that PP DCs induce IgA production (7–9), using freshly isolated, highly purified DCs subsets. Further, IgA production stimulated by PP DCs was largely suppressed by adding anti-IL-6 Ab to the culture. Consistent with the role of IL-6 in IgA production, IgA levels were enhanced in the presence of exogenous IL-6 in the culture when SP DCs were used as the APCs, albeit not restoring it to the levels induced by PP DCs. These data suggest that although IL-6 in the cultures of PP DCs was critical for IgA production, there are factors other than IL-6 that contribute to the observed IgA induction by PP DCs. PP DCs may induce IgA production via another cytokine, such as TGF-β (5), or costimulatory molecules, in addition to IL-6. Several studies have demonstrated that IL-6 is an important factor for the terminal differentiation of IgA B cells in vitro (19, 21) and in vivo (22). Ramsay et al. (22) reported that in mice with targeted disruption of the gene that encodes IL-6, greatly reduced numbers of IgA-producing cells were observed at mucosae. Beagley et al. (26) reported high level IgA-producing B-1 cells poorly responded to additional stimulation with IL-6 in vitro and that a higher frequency of IgA^+ B-1 cells was present in the intestinal lamina propria of $IL\text{-}6^{-/-}$ mice as compared with wild-type mice. These results suggest that IL-6 is critical for IgA production by B-2 cells, but not B-1 cells, and IgA production levels are reduced in $IL\text{-}6^{-/-}$ mice; however, low IgA production is observed in $IL\text{-}6^{-/-}$ mice because B-1 cells induce IgA production in an IL-6 independent manner. On the other hand, Bromander et al. (30) reported that normal total IgA levels in serum and frequencies of IgA plasma cells were found in $IL\text{-}6^{-/-}$ mice and they observed no significant difference in frequencies and distribution of B-1 cells in intestine-associated tissues between $IL\text{-}6^{-/-}$ mice and wild-type mice. Taken together, our data and that of Bromander et al. (30) suggest that there may be several mechanisms of IgA induction including an IL-6-dependent IgA response induced by PP $CD11b^+$ DCs, as well as an IL-6 independent pathway, perhaps occurring in other inductive sites.

PP $CD11c^+/B220^-$ DCs were found to express slightly higher levels of MHC class II, CD86 and CD40 compared with SP DCs (5). In the present study we found that DEC-205 expression was higher on PP DCs compared with SP DCs, particularly in the case of $CD11b^+$ DCs (data not shown). The enhanced expression of these costimulatory molecules and DEC-205 has been shown to correlate with maturation of DCs, so this result suggests that there are a greater number of mature $CD11b^+$ DCs in PP compared with SP. Indeed, PP DCs were found to have enhanced T cell proliferative capacity compared with SP DCs (5), supporting the heightened state of activation of the PP DCs. Because PP $CD11b^+$ DCs are present in the SED, the region just beneath the follicle-associated epithelium containing M cells, they may be constantly being stimulated with the incoming Ags that enter via M cells into PP and are continuously undergoing maturation. PP $CD11b^+$ DCs were capable of secreting higher levels of IL-6 compared with PP $CD8\alpha^+$ DCs and all SP DC populations. It is possible that the high IL-6 secretion was related to their maturation status within the $CD11b^+$ subset.

Our results suggest that $CD11b^+$ DCs may be stimulated by interaction with T cells in the SED and secrete IL-6. Alternatively, a microbial product may induce migration to other sites such as the IFR, as postulated in other studies (10). The migration of $CD11b^+$ DCs may enable these cells to stimulate IgA production more productively in vivo, particularly within the B cell follicles and in the germinal center.

The regulation of IL-6 gene expression in PP DCs is unknown. It has been reported that IL-6 production involves the protein kinase A (31–34), NFAT (31, 35), mitogen-activated protein kinase (31), and AP-1 (31) pathways. Several transcription factors, which bind to the promoter site of IL-6, have been identified. The respective binding of IL-1 inducible factors, NFIL6 (36), NF-κB (36,

37), and IFN regulatory factor (37) to IL-1 responsive element, and the binding of transcriptional factor Sp1 to three repeats of the CCACC element of the IL-6 promoter (36) were found to induce the transcription of IL-6. Therefore, we speculate that the expression or activation of these transcription factors within the PP $CD11b^+$ DCs may be different from other DC populations.

London et al. (38, 39) reported that gut mucosal infection with reovirus stimulates the appearance of virus-specific CTL precursors in the PP. The generation of a CTL response in PP may be important in preferentially repopulating mucosal tissues with effector CTLs that could result in the local containment of infections in the gut. IL-6 was found to function as a late-acting killer helper factor in the differentiation of CTLs (40). It is possible that $CD11b^+$ DCs presenting in SED efficiently enhance not only IgA induction but also CTL induction by secretion of IL-6.

In conclusion, this study demonstrates that PP $CD11b^+$ DCs have a distinct ability to secrete higher levels of IL-6 compared with PP $CD8\alpha^+$ DCs or SP DC populations. Moreover, we demonstrated that IL-6 is a critical factor in PP DCs' ability to induce IgA production. Future studies must investigate the in vivo role of IL-6 secreted by PP DCs in IgA production, including the analysis of in situ IL-6 production by $CD11b^+$ PP DCs during an ongoing PP B cell response involving IgA secretion, and the confirmation of the strict requirement for IL-6 in this process using IL-6 deficient mice.

Acknowledgments

We thank Drs. Sonoko Habu and Takehito Sato for providing OVA-specific TCR Tg mice (OVA 23–3).

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