

Distinct Populations of Dendritic Cells Are Present in the Subepithelial Dome and T Cell Regions of the Murine Peyer's Patch

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Summary

Despite the fact that the Peyer's patch (PP) is the primary site for antigen uptake in the intestine, the cellular basis of antigen handling after transport into the PP is poorly understood. We performed immunohistology of murine PPs using the dendritic cell (DC)-reactive monoclonal antibodies N418, NLDC-145, M342, and 2A1, as well as antibodies to other T cell, B cell, and macrophage markers. N418⁺, 2A1⁺, NLDC-145⁻, M342⁻ cells form a dense layer of cells in the subepithelial dome (SED), just beneath the follicle epithelium, and are scattered throughout the follicle, sparing the germinal center. In contrast, N418⁺, 2A1⁺, NLDC-145⁺, and M342⁺ DCs are present in the interfollicular T cell regions (IFR). CD3⁺ and CD4⁺, but no CD8⁺ T cells were present in the SED and the follicle, including the germinal center, while CD3⁺, CD4⁺, and CD8⁺ T cells were present in the IFR. B cells and macrophages were poorly represented in the SED as no B220⁺ cells, only few Mac-1^{lo} cells, and no F4/80⁺ cells were present at this site. In contrast, Mac-1^{hi} cells were found in the IFR and lamina propria of intestinal villi, while F4/80⁺ cells were found only in the latter. In further phenotypic studies, we analyzed surface molecules of PP and spleen DCs by flow cytometry and found that these cells had similar fluorescence profiles when stained with N418, NLDC-145, and 33D1 DC-reactive antibodies, and antibodies to the costimulatory molecules B7-1 (1G10) and B7-2 (GL1). In contrast, PP DCs expressed 5–10-fold higher levels of major histocompatibility complex class II antigens (IE^b) than spleen DCs. Finally, in functional studies, we demonstrated that both PP and spleen DCs process soluble protein antigens during overnight culture and induce similar levels of proliferation in CD3⁺ T cells, and CD4⁺/Mel 14^{hi} T cells from T cell receptor transgenic mice. The *in vivo* relevance of such presentation was shown by the fact that PP DCs isolated from Balb/c mice after being fed ovalbumin stimulated proliferation in ovalbumin T cell receptor T cells. Taken together, our data suggest that DCs in the SED of the PP are uniquely positioned for the processing of antigens passed into the PP from the overlying M cell, and that PP DCs are effective at processing and presenting oral antigens to naive T cells.

The Peyer's patch (PP)¹ is the primary site for antigen processing in the intestine. While it is clear that luminal antigens gain access to this site via transfer across specialized epithelial cells, known as M cells (microfold cells), that are scattered among the columnar epithelial cells above the PP dome, the cellular basis of antigen processing after transport into the PP is poorly understood.

As is the case with other epithelial cells, M cells themselves express MHC class II antigens and IL-1 (1, 2). Their antigen-presenting function, however, is likely to be limited by their physical isolation from the bulk of the T cell population. Macrophages have been thought to be in-

volved in this function, and indeed macrophages have been demonstrated in the subepithelial dome (SED) of the PP by electron microscopy (3) and are presumed to be the cells responsible for the uptake of carbon in mice chronically fed carbon in their drinking water (4). Another candidate antigen-presenting cell is the so-called pocket lymphocyte, a poorly characterized lymphoid cell present within the confines of the antiluminal membrane of the M cell. Both soluble molecules, such as horseradish peroxidase (5), and viable microorganisms, such as *Salmonella typhimurium* (6), have been shown to be taken up by these cells after entering the PP in mice. In the rabbit, pocket lymphocytes are MHC class II positive, and surface IgM, CD3, CD4, and CD25 negative (7).

In addition to macrophages and M cell pocket lymphocytes, MHC class II-positive cells with the morphology of

¹Abbreviations used in this paper: ABC, avidin-biotin complex; DC, dendritic cells; IFR, interfollicular regions; LP, lamina propria; M cells, microfold cells; PP, Peyer's patch; SED, subepithelial dome.

dendritic cells (DC)s have been identified in the SED, as well as in the interfollicular (T cell) region (IFR) of the PP (8), and DCs that stain with the monoclonal antibodies N418 (anti-CD11c) and M342 (anti-DC) have been described in the PP of osteopetrotic mice that are deficient in macrophage CSF (9). In addition, there is one recent immunohistologic study of human PPs that identified the presence in the SED region of MHC class II⁺, lysozyme⁻, S-100 protein⁺ cells with cytoplasmic processes that extended into the dome epithelium that are most likely DCs (10).

In this report, immunohistology of murine PPs was performed using the DC-reactive mAbs N418, NLDC-145, M342, and 2A1, as well as antibodies to other T cell, B cell, and macrophage markers. We demonstrated the presence of two populations of PP DCs, one of which forms a dense layer just beneath the dome epithelium and another phenotypically distinct population in the IFR. Further, we enriched these cells by transient plastic adherence and flow cytometric sorting for N418⁺ cells, described their surface phenotype by flow cytometry, and showed that PP DCs are capable of inducing proliferation in T cells after both in vitro and in vivo antigen loading. We suggest that the DC population in the SED is uniquely positioned for the processing of antigens passed into the PP from the overlying M cell.

Materials and Methods

Animals. Female B10.A and Balb/c mice 6–10 wk of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice transgenic for the V α 11/V β 3 TCR that recognizes the 88–104 COOH-terminal peptide of pigeon cytochrome c and IE^k were originally provided by Ronald Schwartz (Laboratory of Cellular Immunology, National Institute for Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD). The F-1 line used in these experiments was maintained by backcrossing B10.D2 (H-2^d) mice heterozygous for the TCR alpha and beta chains, with B10.A (H-2^k) mice (The Jackson Laboratory). Mice homozygous for a TCR recognizing the 323–339 peptide of ovalbumin and IA^d in a Balb/c background were kindly provided by Dennis Loh (Howard Hughes Medical Institute, Washington University, St. Louis, MO).

Preparation of DCs and Antigen Loading Protocols. DCs were prepared with a modification of established techniques (11). PP were dissected and digested with collagenase D (400 Mandl U/ml; Boehringer Mannheim, Mannheim, Germany) and DNase (15 μ g/ml DNase I, Boehringer Mannheim) in 10 ml IMDM (Gibco/BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, penicillin/streptomycin, 2-mercaptoethanol (50 μ M), L-glutamine (2 mM), and Amphotericin B (0.5 μ g/ml, FungizoneTM; Gibco/BRL) for 30 min at 37°C. Released cells were plated on tissue culture dishes (3025; Falcon Labware, Oxnard, CA) in supplemented RPMI 1640 for 60 min at 37°C. Nonadherent cells were washed free with warm PBS, and adherent cells were cultured for 18 h with supplemented RPMI 1640 in the presence or absence of 100 μ g/ml pigeon cytochrome c (Sigma Chemical Co., St. Louis, MO), or 1 mg/ml ovalbumin (Sigma Chemical Co.). DC-enriched, nonadherent cells were recovered by washing the plastic dishes with warmed PBS. By flow cytometry these cells were 65–90% N418⁺, 10–25% B220⁺, and

2–4% CD3⁺. Spleen DCs were prepared in an identical fashion, and by flow cytometry were 70–95% N418⁺, 10–18% B220⁺, and 1–3% CD3⁺. Highly enriched N418⁺ cell populations were prepared from nonadherent cell populations by flow cytometric sorting for N418⁺ cells; the sorted cells were 98–99% N418⁺. Whole PP cell populations yielded 0.1–0.5% DCs, whereas whole spleen populations yielded 0.5–1.2% DCs.

For studies with in vivo antigen loading, three doses of 250 mg ovalbumin in 0.5 ml of PBS and 100 μ l of a 2.5% solution of 0.5–0.75- μ m latex beads (Polysciences, Warrington, PA) was given by gavage to each of 15 Balb/c mice. The first two doses were separated by 15 h and the last dose was given after an additional 3 h. In addition, the animals' drinking water was supplemented with 5 mg/ml ovalbumin. Mice fed only microbeads served as controls. Mice were given no solid food throughout the period of oral antigen dosing. PP DCs were isolated 3 h after the last feeding by transient plastic adherence and used to stimulate transgenic T cells as indicated.

Preparation of T Cells. T cells were prepared by negative selection on immunoaffinity (anti-mouse Ig) columns (Isocell[®] mouse T cell column; Pierce Chemical Co., Rockford, IL) from lymph nodes or spleens of 8–16-wk old TCR transgenic mice according to the manufacturer's instructions. The purity and transgene expression of the T cell preparations was verified by dual color flow cytometry. Resulting cell populations were 85–90% CD3⁺, and 5–10% B220⁺; 70–90% of CD3⁺ cells also expressed V α 11 or the KJ1-26 clonotype. CD4⁺, Mel-14^{hi} T cells were isolated by two-color flow cytometric sorting and were routinely 98–99% positive for the two markers.

Procedure for Immunoperoxidase Staining of Frozen Sections. PPs were dissected, placed in OCT freezing medium (Miles Laboratories Inc., Elkhart, IN), and flash frozen. 10- μ m acetone-fixed sections were stained as follows: (a) After quenching endogenous peroxidase activity, tissue sections were rehydrated in PBS containing 0.1% BSA (fraction V, PBS/BSA; Sigma Chemical Co.), and then blocked for 30 min with 2% mouse, and 3% goat or rabbit serum (same species as secondary antibody); (b) blocking solution was removed and the tissue sections were incubated for 60 min with primary or control antibodies, prepared in PBS with 1% normal mouse serum and 2% goat or rabbit serum (PBS serum); (c) sections were washed in PBS/BSA and then incubated for 30 min with biotinylated secondary antibody in PBS-serum; (d) tissue sections were washed for 30 min in three changes of PBS/BSA, and then incubated for 30 min with avidin-biotin complex (ABC) linked to horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) prepared according to the manufacturer's instructions; (e) after washing for 30 min in six changes of PBS/BSA, tissue sections were incubated with 0.05% (wt/vol) 3,3-diaminobenzidine (Sigma Chemical Co.), 0.05% NiCl (wt/vol), and 0.03% H₂O₂; (f) sections were rinsed, counterstained with 5% methyl green (wt/vol) (Fisher Scientific, Fair Lawn, NJ) in methanol, rinsed in distilled water, and permanently mounted with Permount (Fisher Scientific).

Antibodies. Monoclonal hamster anti-mouse CD11c (N418) (12), rat anti-Cd11b (MAC-1, M1/70) (13), rat antimacrophage (F4/80) (14), hamster anti-MHC II (M5), and rat anti-DC (33D1) (15) were obtained from American Type Culture Collection (Rockville, MD). Rat anti-mouse IDC (NLDC-145) (16) was kindly provided by George Kraal (Free University, Amsterdam, The Netherlands). The monoclonal mouse-DC-reactive hamster antibody M342 (17) and rat antibody 2A1 (18) were kindly provided by R. M. Steinman (The Rockefeller University, New York). The biotinylated monoclonals anti-B220 (RA3-

6B2.1) (19), anti-CD4 (RM4-4), anti-CD8 (53-6.7), anti-CD3 ϵ (2C11) (20), anti-B7-1 (1G10) (21), anti-B7-2 (GL1) (22) and anti-LECAM-1 (Mel-14) were purchased from PharMingen (San Diego, CA). Normal rat serum, and normal hamster serum (1:1,000 dilution) (Sigma Chemical Co.) served as control antibodies, along with 2C11, a hamster mAb that does not react with DCs. For flow cytometry, FITC-labeled goat F(ab')² anti-hamster IgG, and FITC-labeled goat anti-rat IgG were purchased from Caltag (South San Francisco, CA), and directly labeled

FITC anti-CD3 (2C11), FITC anti-B220 (RA3-6B2.1), biotinylated anti-V α 11 TCR, and PE-streptavidin were purchased from Pharmingen. KJ1-26 (23) was kindly provided by K. Nakayama in Dennis Loh's laboratory.

Flow Cytometry and Flow Cytometric Sorting. To block nonspecific FcR binding of the primary antibody, DC preparations were initially treated with anti-FcR γ II mAb (2.4G2, for hamster primary antibodies), or with 5% mouse serum (for rat primary antibodies). This incubation was followed by FITC-labeled B220, or

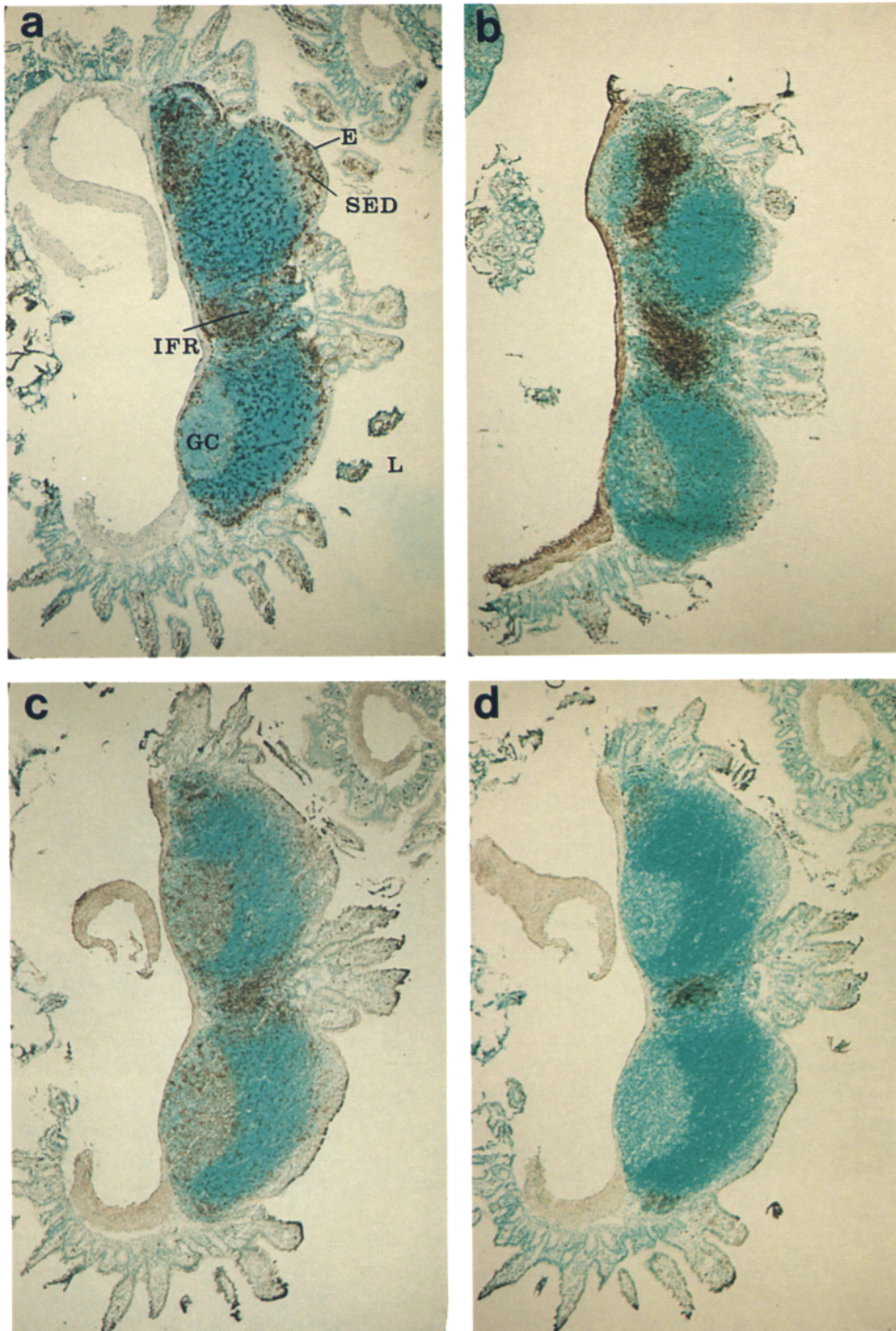
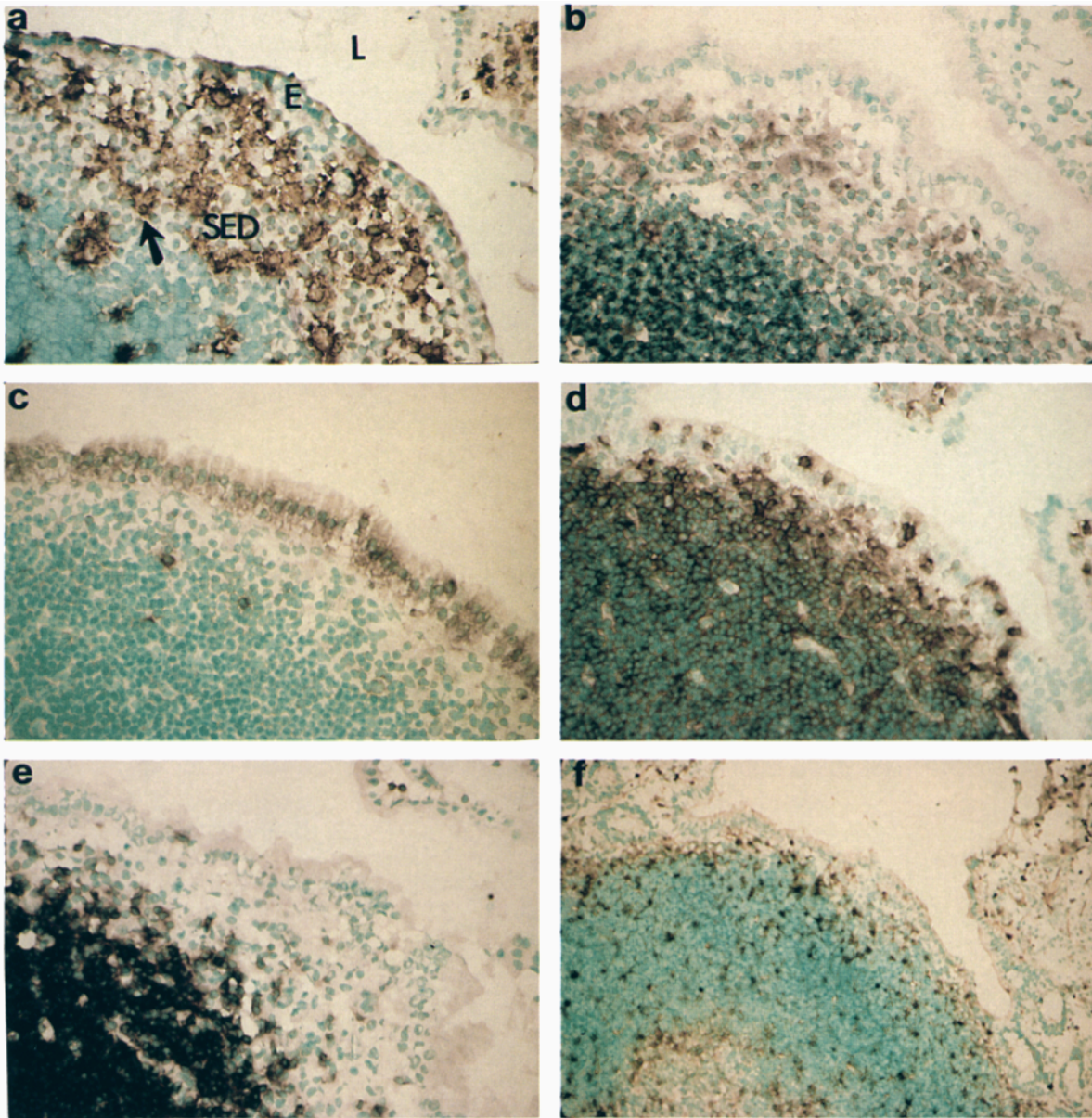


Figure 1. N418⁺ DC are located in the subepithelial dome of the PP. Immunohistology of frozen sections of a PP dissected from an 8-12-wk-old B10.A mouse. Sections were stained with the ABC technique and counterstained with methyl green. (a) Anti-CD11c (N418). (b) Anti-CD3 (2C11). (c) Anti-CD4 (RM4-4). (d) Anti-CD8a (53-6.7). L, lumen; GC, germinal center; SED, subepithelial dome; IFR, interfollicular region; E, epithelium.



N418, 33D1, NLDC-145, M5, or 2C11 mAbs, in the form of cell culture supernatants, and PE-labeled F(ab')₂ goat anti-hamster or goat anti-rat IgG (Caltag). Flow cytometry was performed on a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA). FACS® sorting for N418⁺ cells was performed on a FACStar® sorter (Becton Dickinson).

Proliferation Assay. Purified cytochrome c or ovalbumin TCR T cells were cultured with varying numbers of purified DCs as indicated in round bottom 96-well tissue culture plates (Nunc, Roskilde, Denmark) in supplemented RPMI 1640 media for 48 h. During the last 8 h of culture, 1 μCi/well of [³H]thymidine (Amersham Corp., Arlington Heights, IL) was added, cells were frozen, and subsequently harvested (PHD harvester; Cambridge Technologies, Watertown, MA) and counted in a beta emission scintillation counter. All cultures were set up in triplicate with values expressed as mean values ± standard deviation of the mean.

Results

N418⁺ Dendritic Cells Are Concentrated in the IFR and Subepithelial Dome. In initial studies, frozen sections of mouse PP were stained with the anti-CD11c mAb N418 using an immunoperoxidase technique. This mAb has been shown previously to identify a surface antigen present on most (if not all) DC (12). As shown in Fig. 1 A, N418⁺ cells formed a dense layer of cells located just beneath the epithelial cells in the SED; in addition, N418⁺ cells were found in the IFR and scattered throughout the rest of the PP, but were conspicuously absent from the germinal center. Of interest, as shown in Fig. 1, B and C, only small numbers of CD3⁺ and CD4⁺ T cells were present in the subepithelial dome, but such cells were present in high concentration in the IFR, as well as in low concentration throughout the PP including the germinal center. Finally,

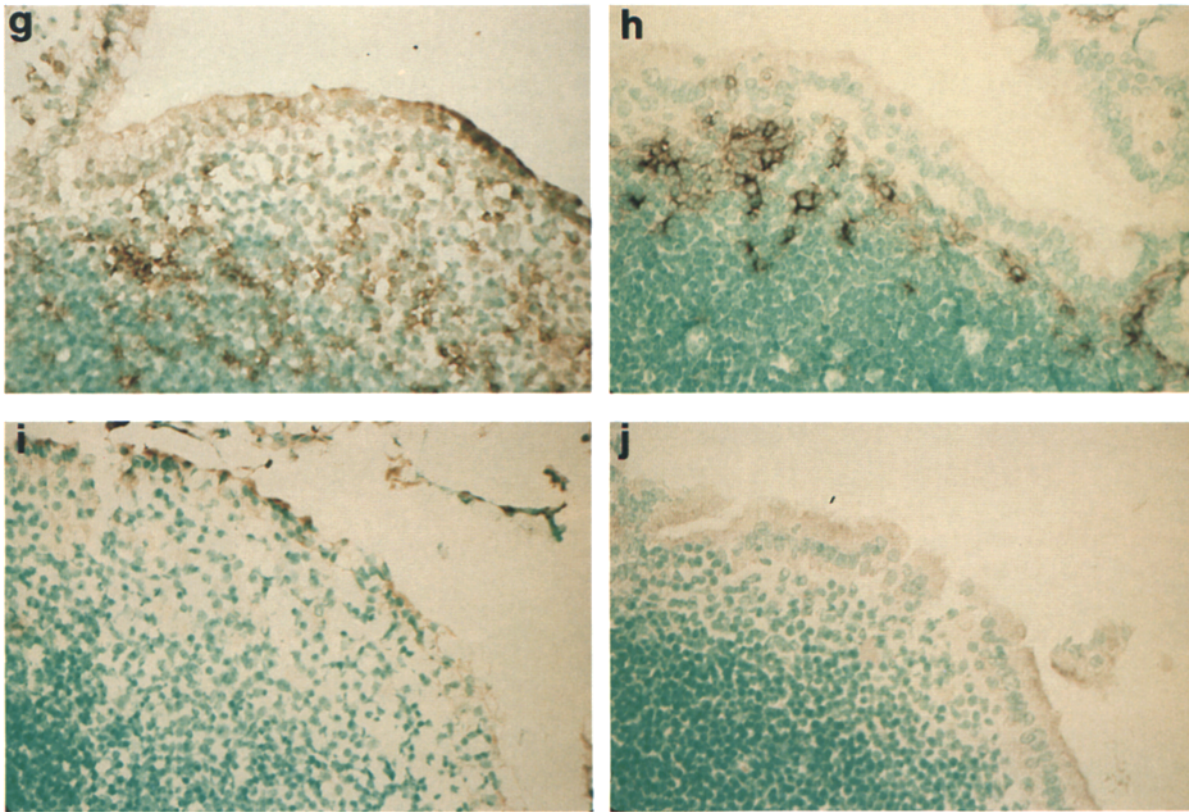


Figure 2. Subepithelial N418⁺ DCs appear to be MHC class 2⁺, 2A1⁺, MAC-1^{lo}, and are not T or B cells. Immunohistology of the subepithelial dome region of a PP. Sections were stained with the ABC technique and counterstained with methyl green. (a) Anti-CD11c (N418). (b) Anti-DC/B cell (2A1). (c) Anti-IDC (NLDC-145). (d) Anti-MHC II (M5). (e) Anti-B220 (RA3-6B2). (f) Anti-CD3 (2C11). (g) Anti-CD4 (RM4-4). (h) Anti-CD11b (Mac-1, M1/70) (i) Control rat IgG (serum 1:1,000). (j) Control hamster IgG (serum 1:1000). L, lumen; SED, subepithelial dome; E, epithelium. Note the presence of both CD4⁺ and N418⁺ cells in the SED.

as shown in Fig. 1 D, CD8⁺ cells appeared in a discrete area of the IFR, and not in the SED, or the follicle.

Subepithelial N418⁺ DCs Are MHC II⁺, 2A1^{lo}, Do Not Stain with T or B cell-specific mAbs, and Are Phenotypically Distinct from DCs in the Interfollicular Region. As shown in Fig. 2, the immunoperoxidase-stained SED area of PPs visualized under high power (×40) light microscopy disclosed a band of N418⁺ cells just under the epithelium that display dendritic morphology (Fig. 2 A, arrow). In addition, as shown in Fig. 2 B, cells corresponding in position to the N418⁺ cells stained with mAb 2A1, an mAb previously shown to bind to a DC and B cell intracellular antigen (18). In contrast, as shown in Fig. 2 C, mAb NLDC-145, an mAb previously shown to stain interdigitating DCs in peripheral lymph nodes (16), reacted only with an occasional cell in the SED; similarly, as shown in Fig. 3 C, M342, an mAb previously shown to bind to an intracellular antigen of DC and some B cells (17), did not react with any of the cells in the SED. Finally, as shown in Fig. 2 D, an mAb specific for MHC class II revealed diffuse staining throughout the PP, but with concentrated staining in B cell areas of the follicles and in the SED, the latter consistent with the distribution of DCs in the SED. Intraepithelial cells in the PP dome were stained for MHC class II, but only occa-

sionally for N418 (not shown), consistent with the staining of pocket lymphocytes.

The vast majority of the cells in the SED expressing MHC class II were not B cells or macrophages. Thus, as shown in Fig. 2 E, very few cells in the SED stained for B220, and, as shown in Figs. 2 H, and 3, E and F, only a modest number of MAC-1 (CD11b)^{lo} and no F4/80⁺ cells (macrophage markers) were found in this area.

As noted above, N418⁺ DCs were found in the IFRs as well as in the SED area. However, as shown in Figs. 2 and 3, while the SED DCs were stained positive for N418 and 2A1, and negative for NLDC-145 and M342, the IFR DCs were positive for all four markers (Fig. 3, A–D). Thus, SED DCs do not express at least two antigens expressed by IFR DCs.

Distribution of Cells Expressing Macrophage-associated Antigens in the Intestinal Mucosa. Cells expressing macrophage markers were either few and lightly stained (MAC-1) or not present at all (F4/80) in the DC-enriched area in the SED, but were present in the IFR. Thus, as shown in Fig. 3, cells prominently expressing Mac-1 were present throughout the IFR and a few cells expressing F4/80 were present near the serosa of the IFR. In addition, cells positive for both markers were present in the lamina propria

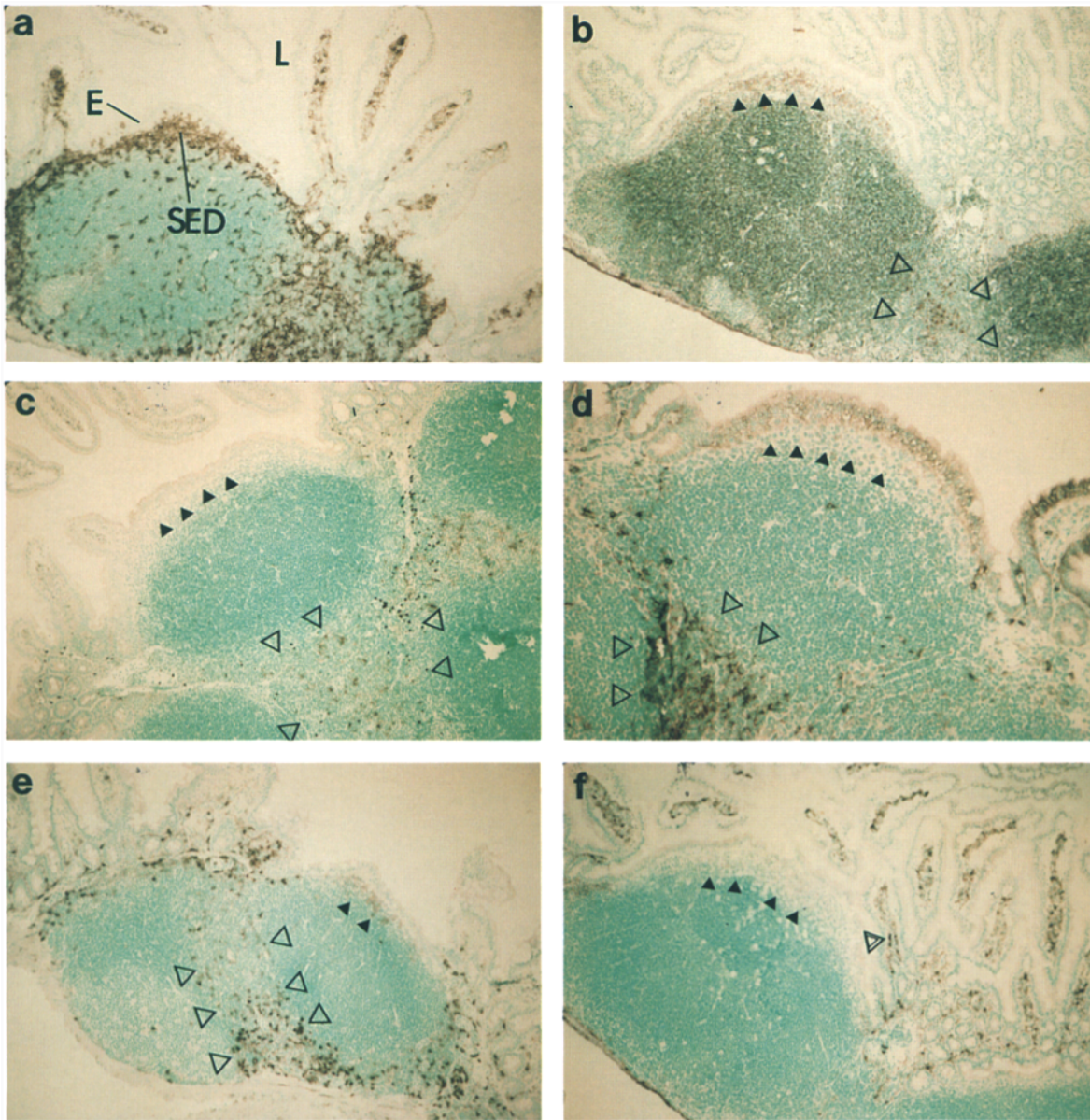


Figure 3. Subepithelial DCs are phenotypically different from DCs in the IFR of the PP. Immunohistology of frozen sections of a PP dissected from an 8–12-wk-old B10.A mouse. Sections were stained with the ABC technique and counterstained with methyl green. Small dark arrowheads emphasize cells in the SED (*b*, *e*, and *f*) and large light arrowheads emphasize cells in the T cell regions (*c–f*). The double arrow in *f* indicates cells in the LP of an intestinal villus. (*a*) Anti-CD11c (N418). (*b*) Anti-DC/B cell (2A1). (*c*) Anti-DC/B cell (M342). (*d*) Anti-IDC (NLDC-145) (*e*) Anti-CD11b (MAC-1, M1/70). (*f*) Anti-macrophage (F4/80).

(LP). Therefore, it appears that macrophages have an overlapping distribution relative to DCs; DCs, and few, if any, macrophages are present in the SED, while both DCs and macrophages are present in the IFR of the PP and in the LP. These findings must be qualified, however, since MAC-1 (anti-CD11b) may react with cells other than macrophages (e.g., DCs or NK cells). The Mac-1 staining in the IFR is therefore not definitive for the presence of macrophages at this site.

PP DCs Express 5–10-fold Higher Levels of MHC Class II Antigens Than Spleen Dendritic Cells. In further studies, we purified a population of PP DCs using a technique based

on the fact that DCs are transiently adherent to plastic, a procedure previously used for the isolation of spleen DCs (11). We compared the surface phenotype of spleen and PP DCs isolated in this fashion to determine if PP DCs had particular surface characteristics that may help explain the unique immune responses of the mucosa. As shown in Fig. 4, spleen and PP DCs had similar flow cytometry profiles for staining with the DC-reactive antibodies N418, NLDC-145, and 33D1. In addition, both PP and spleen DCs expressed similar high levels of B7-1 and B7-2 antigens (Fig. 4 *B*). In contrast, when compared to spleen DCs, PP DCs expressed a 5–10-fold higher level of MHC class II

antigens (IE^k).

PP DCs Process Soluble Protein Antigens Both In Vitro and In Vivo for Presentation to Naive T Cells from TCR Transgenic Mice. In a final series of experiments, the ability of purified PP DCs to present antigen was tested in a T cell proliferation assay. For this purpose, T cells from mice transgenic for a TCR specific for a cytochrome c peptide and IE^k were cultured with purified DCs pulsed with intact cytochrome c during the overnight culture required for their isolation. As shown in Fig. 5, PP DCs isolated by transient plastic adherence and flow cytometric sorting for N418⁺ cells induced proliferation equivalent to that of similar pop-

ulations isolated from the spleen. This was shown for two separate T cell populations (from LN and spleen) for the FACS[®] purified N418⁺ cells (Fig. 5 A) and at different DC/T cell ratios for the crudely purified cells (not shown). To provide additional evidence that PP DCs were able to stimulate naive T cells, we purified CD4⁺/Mel-14^{hi} cells from ovalbumin TCR transgenic mice and demonstrated that with this population of T cells, PP DCs and spleen DCs loaded with ovalbumin in vitro were also equally capable of inducing T cell proliferation (Fig. 5 B).

Lastly, we demonstrated that PP DCs could be loaded in vivo by antigen feeding. For these studies, after feeding ovalbumin to normal Balb/c mice, we isolated PP DCs by transient plastic adherence, and then determined the ability of the isolated cells to stimulate ovalbumin TCR T cells in vitro in the absence of additional ovalbumin. As seen in Fig. 6, PP DCs from fed mice stimulated ovalbumin TCR T cells to proliferate (stimulation index [SI] = 6, *P* < 0.001) whereas PP DCs isolated from unfed mice lacked this capacity.

Discussion

Antigens from the intestinal lumen are transported across M cells into the SED of PP where initial antigen presentation to T cells is likely to occur. Using immunoperoxidase staining of frozen sections of murine PPs, we demonstrated the presence of a dense layer of cells just beneath the dome epithelium with dendritic morphology that stain with anti-murine CD11c (mAb N418). Additional staining suggested that these cells express MHC class II and an intracellular antigen of DCs and B cells recognized by mAb 2A1. A

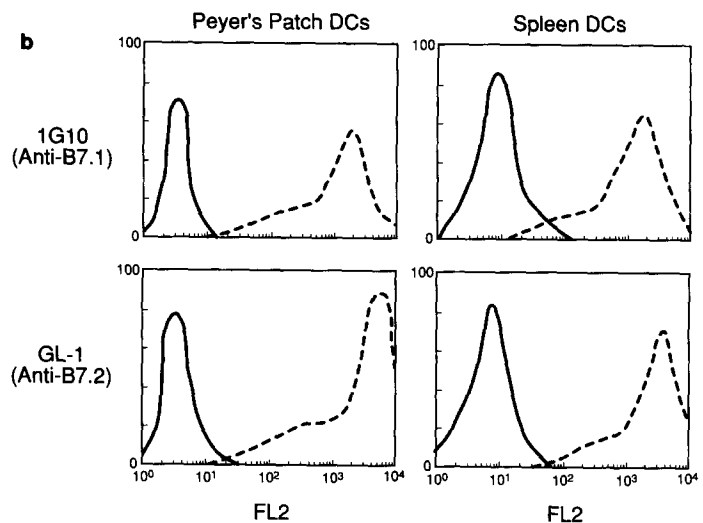
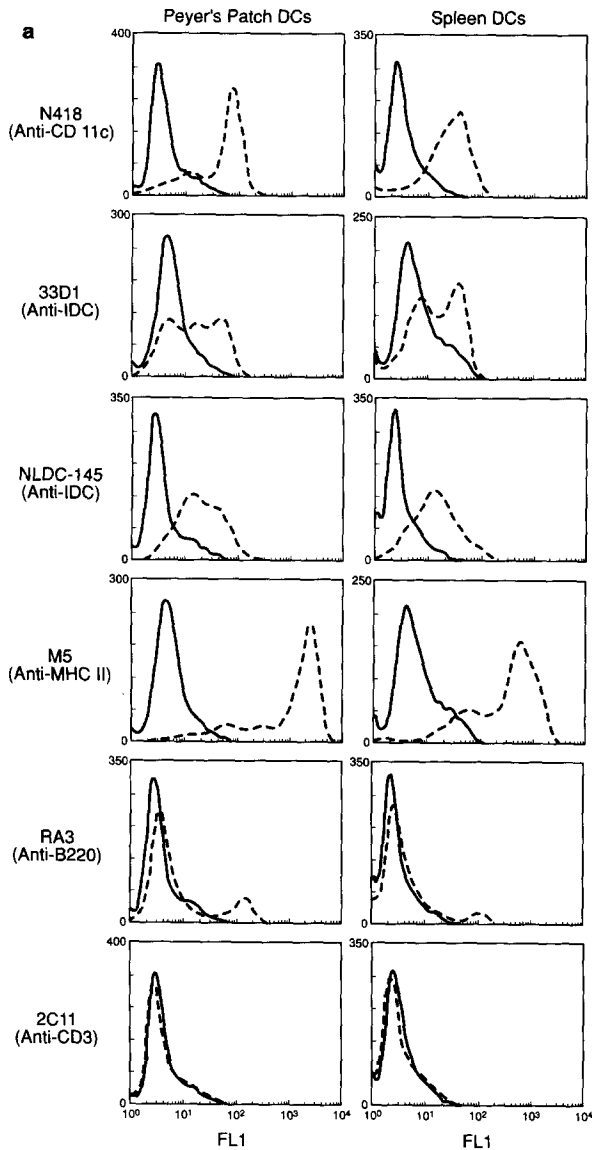


Figure 4. Flow cytometry of DCs purified by transient plastic adherence. Cells from the PP or spleen were isolated by transient plastic adherence (i.e., after 18 h of culture) and stained with the mAbs indicated on the left, followed by an FITC-labeled, F(ab')₂ goat anti-hamster or anti-rat IgG. The results are shown as histograms with fluorescence intensity on the x-axis and cell number on the y-axis. The solid graph represents control hamster or rat IgG and the broken graph, staining with the stated mAb. No cells were excluded from analysis on the basis of forward or side scatter for cells in *a*. In analyses depicted in *b*, cells were dual stained with N418 and anti-hamster FITC, and with biotinylated anti-B7-1 or anti-B7-2 followed by streptavidin PE. Histograms depict B7-1 or B7-2 staining of N418⁺ cells. Staining of PP and spleen DC preparations were done simultaneously and results are representative of two experiments done with similar results.

subpopulation may also express low levels of CD11b/MAC-1, characteristic of DCs from other organs. While there were CD3⁺ and CD4⁺ cells in this region as well, the pattern of staining did not suggest that the N418⁺ cells were T cells, nor that they expressed significant levels of CD4 or CD8, as has been demonstrated for populations of spleen (24), blood (25), or skin-derived DCs (26). Finally,

B220⁺ cells were not found in the area just beneath the dome epithelium, although they were readily stained in the underlying follicle. Taken together, these findings strongly argue that the PP is characterized by a layer of DCs which are poised for the capture and presentation of gut luminal antigens to PP T cells.

There is now extensive evidence that the N418 mAb reacts with murine CD11c, which is found primarily on dendritic cells in the mouse. It does not react with peritoneal macrophages, splenic B lymphoblasts, or spleen or lymph node lymphocytes (12), and has been used to FACS[®] sort for dendritic cells from the mouse spleen (24). Thus, the N418 mAb recognizes an antigen on the surface of cells having other well-recognized characteristics of DCs, but does not recognize freshly isolated macrophages, B cells, or T cells. Therefore, the combined evidence from previous studies, as well as the present study, suggest that the N418⁺ SED cells are, in fact, DCs.

The subepithelial population of DCs appeared to be different from those present in the IFR of the PP, which were typical of the interdigitating DCs described by others (16, 17). Thus, the SED DCs were N418⁺/2A1⁺, but NLDC-145⁻/M342⁻, while the IFR DCs reacted with mAbs to all four markers. These results are consistent with those obtained in a recent study of the antigens recognized by N418 and M342 mAbs in tissue sections of a PP from the osteopetrotic mouse, which is deficient in macrophage colony CSF (9). The authors found a broad expression of N418 antigen on cells surrounding the follicle and a limited expression of M342 antigen on cells in the IFR. Staining of the SED, however, was not examined. Our results are also consistent with a prior study demonstrating NLDC-145 staining of intestinal epithelial cells and the IFR, but not the lymphoepithelium of the PP (27).

Based on the information of the current and prior studies, it appears quite likely that there are at least two different populations of DCs in the PP. One is a population of interdigitating DCs in the IFRs that is similar to that found in the paracortex of the peripheral lymph nodes or in the periarteriolar lymphocyte sheaths of the spleen. The other is similar to an N418⁺, NLDC-145⁻, M342⁻ population of spleen DCs that form a dense network in the periphery of the white pulp, where they are in the direct path of migrating T cells (12, 17). In addition, it can be argued that the M342⁻ cells of the PP SED are in a less differentiated state than the M342⁺ DCs of the PP IFR, since freshly isolated M342⁻ spleen DCs acquire this antigen (17), along with high levels of MHC antigens, costimulatory molecules, and antigen-presenting functions upon overnight culture (12, 28).

The MAC-1/Cd11b^{lo} staining present in the SED could be due to the presence of macrophages at this site. However, it is more likely that the MAC-1^{lo} cells found in the SED are DCs, since this antigen is present at low levels on DCs from other organs (11) and in addition, we found a lack of CD11b^{hi} and F4/80⁺ cells, or cells that have intrinsic peroxidase activity (data not shown), i.e., cells with staining properties typical of macrophages, in the SED. On

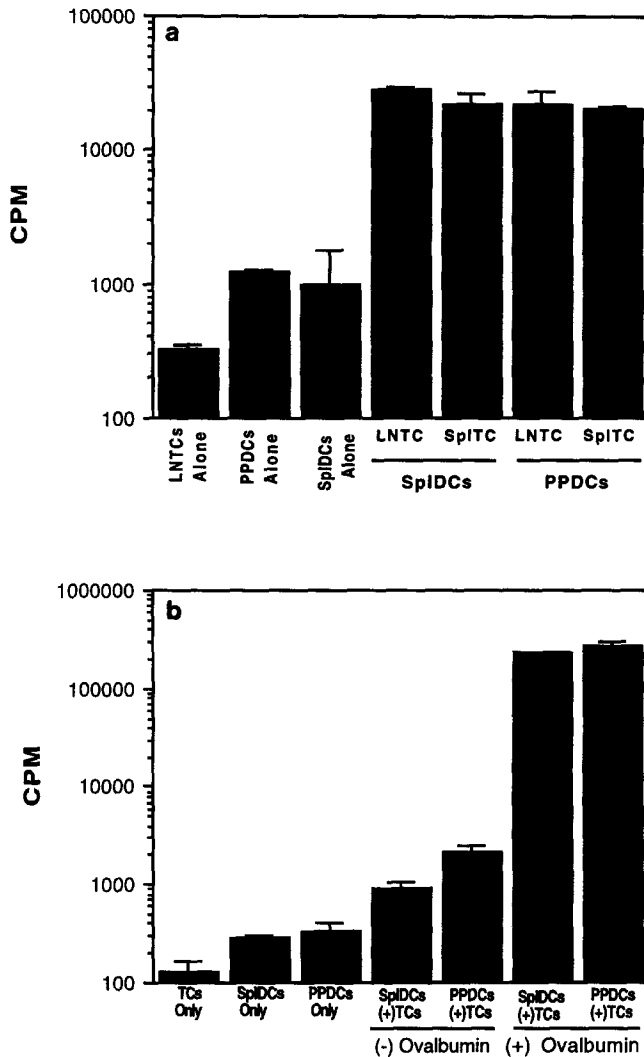


Figure 5. Proliferation of T cells from cytochrome C or ovalbumin TCR transgenic mice. Proliferation was measured by [³H]thymidine uptake during the last 8 h of a 48-h culture. Results are presented as the mean cpm of triplicate cultures \pm standard deviation. (a) DCs purified by flow cytometric sorting for N418⁺ cells. 5×10^4 purified lymph node T cells (LNTC) or spleen T cells (SplITC) were stimulated with 5×10^3 cytochrome c-pulsed N418⁺ DCs purified by flow cytometry sorting transiently adherent cells. $P < 0.001$ for both SplDCs and PP DCs plus LNTC or SplITC when compared to DCs or T cells alone. Results are representative of three separate experiments conducted with similar results. (b) CD4⁺/Mel-14^{hi} T cells. 10^5 flow cytometry-sorted CD4⁺/Mel-14^{hi} ovalbumin TCR T cells (TCs) were stimulated in vitro by 5×10^4 PP (PPDCs) or Spl DCs (SplDCs). DCs were unsorted, transiently adherent DCs cultured overnight either in the presence (+) or absence (-) of ovalbumin. $P < 0.0001$ when comparing T cell stimulation with the antigen-loaded and unloaded DCs from either the PP or spleen. Results are representative of two separate experiments conducted with similar results.

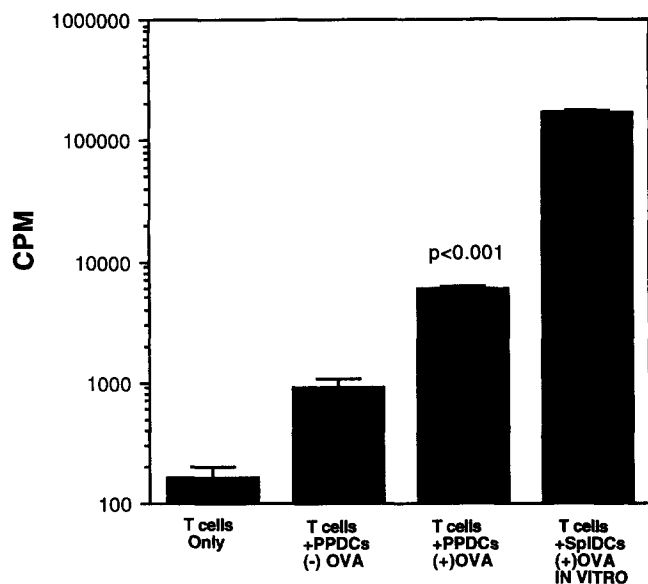


Figure 6. In vivo loading of PP DC with ovalbumin. After feeding ovalbumin to normal Balb/c mice PP DCs were isolated after overnight culture by transient adherence and used to stimulate CD3⁺ spleen T cells from ovalbumin TCR transgenic mice in the absence of additional ovalbumin. 2×10^4 PP DCs from either control mice [(-)OVA] or those fed ovalbumin [(+)OVA] were added to 10^5 T cells and proliferation was measured after 48 h. An equivalent number of T cells were stimulated by 2×10^4 spleen DCs (SplDCs) which had been loaded in vitro with ovalbumin (1 mg/ml) during the overnight incubation [(+) OVA IN VITRO] as a positive control. $P < 0.001$ when comparing values from fed and unfed mice. Results are representative of two separate experiments conducted with similar results.

the other hand, CD11b^{hi} cells were readily apparent in the IFR and the LP, and F4/80⁺ cells were found in the LP, indicating that cells with macrophage-staining properties could be detected in the tissues examined. There was, in fact, a relative paucity of cells with macrophage-staining properties in the PP as a whole, especially since, as mentioned above, other cell types, such as DC or NK cells, may be contributing to the MAC-1 staining in the IFR. This correlates with the observation that the yield of macrophages from isolated PPs using plastic adherence techniques is low compared to that from the spleen, and is consistent with prior studies of immunohistology of murine PP using the F4/80 mAb (14).

In contrast to the findings of others (29), we found that PP DCs could be enriched by transient plastic adherence to 65–90% purity. This population could be further enriched to 99% purity by flow cytometric sorting for N418⁺ cells. By light microscopy, the cells obtained by this technique were morphologically homogeneous and displayed irregular and nondistinct cell margins, features typical of DCs from other organs. Of interest, the N418⁺ PP DCs were larger (higher forward scatter) than spleen DCs (not shown).

The major phenotypic difference between spleen and PP DCs was a 5–10-fold higher level of expression of MHC class II antigens (IE^k on cells from B10.A mice) on PP DCs.

This increased expression of MHC class II may reflect differences in the intrinsic microenvironments of the PP and spleen, or in responses to cytokines and surface signals during the overnight culture required for DC isolation. While the increased expression of MHC II was not accompanied by an increased ability to induce T cell proliferative responses, the density of MHC peptide surface expression may influence T cell phenotype (Th1 vs. Th2) of the stimulated cells by altering the T cell-APC interaction (30). Virtually all of the purified PP DCs expressed the antigens recognized by NLDC-145 and N418. This may be due to the fact that overnight culture of PP DCs, including those from the SED, induces differentiation into cells expressing the phenotype of IFR DCs. Alternatively, it is possible that only IFR DCs are isolated by transient plastic adherence. In any case, it will be necessary to isolate fresh PP DCs for analysis to address the issue of whether NLDC-145⁻ PP DCs differentiate into NLDC-145⁺ DCs in vitro.

Finally, we demonstrated that PP DCs can process soluble antigens (cytochrome c or ovalbumin) both in vitro and in vivo, and present the respective peptides to TCR transgenic T cells. In in vitro studies, we showed that both enriched and purified N418⁺ cells from the PP processed antigens during the first 18 h of culture and induced significant T cell proliferation that was antigen specific, dose (DC:T cell ratio) dependent, and equivalent to that of DCs from the spleen. Since only 80–90% of the T cells from these mice carry the transgenic TCR, and of these, only 70–80% are Mel-14^{hi} (naive; data not shown), these experiments could not distinguish between proliferation of naive and memory T cells. We therefore repeated these studies with purified CD4⁺ cells of the Mel-14^{hi} phenotype and showed that ovalbumin-TCR T cells were stimulated to proliferate by ovalbumin-pulsed PP DCs. In complementary in vivo studies we demonstrate that ovalbumin given orally will be naturally processed by in the PP by PP DCs. Here, we demonstrated that PP DCs loaded by in vivo feeding induced highly reproducible proliferation of ovalbumin TCR transgenic T cells. While the amount of proliferation was relatively low compared to that observed in vitro, we feel this may be highly significant in view of the fact that the GI tract efficiently degrades fed antigen, and the fact that such low level stimulation would be important in the face of continuous antigen feeding. As discussed above, however, it must be emphasized that in these functional studies, we cannot distinguish between antigen presentation by the SED and the IFR DCs. Therefore, while the SED DCs are positioned to capture antigens from the intestinal lumen, we cannot conclude that they are responsible for the T cell proliferation demonstrated in either the in vitro or in vivo antigen loading experiments.

The present investigation of PP DCs complements a small number of previous functional studies of this cell type. In initial studies, PP accessory function was measured by the proliferation of periodate-treated T cells and was shown to be present in a non-B cell, non-T cell, Fc receptor⁻, Ia⁺ PP population with low buoyant density and dendritic morphology (25). In more recent studies, PP

DCs enriched by clustering to irradiated, periodate-treated T cells were shown to be capable (29), and even necessary (31) for inducing T cell help for IgA production by B cells. Finally, low density, fibronectin nonadherent PP cells enriched in PP DCs were shown to be effective stimulators in a mixed lymphocyte reaction (32). In aggregate, these studies, together with the present data, show that PP DCs are important APCs for both T cell proliferation and T cell help of B cell responses in the PP.

In addition to understanding the mechanisms of specific T cell responses and of IgA B cell development in the PP, knowledge of how antigens and microorganisms are handled in the PP is important for understanding the pathogenesis of infectious diseases that gain entry through the PP. In a recent study, mouse mammary tumor virus was demonstrated to infect PP lymphocytes of suckling mice before systemic spread of the infection (33). DCs in the SED could play a role in the spread of such infections by providing the correct environment for initial viral replica-

tion. In fact, it has been recently demonstrated that DC-T cell clusters infected with HIV-1 develop into large syncytia composed of both DC and T cell membranes that produce large amounts of virus (34). Whether intestinal infection with HIV occurs via a route similar to mouse mammary tumor virus, however, is not known.

To conclude, the results of this study provide evidence that the DC network in the SED of PPs is an important component in the uptake and processing of luminal antigens. Such uptake may occur by endocytosis, or even by phagocytosis, as has been demonstrated for Langerhans' cells (35) and DC precursors grown from bone marrow (36). The DCs then present the processed antigen to CD4⁺ T cells in the SED or follicle, or after maturation and migration to the IFR, to CD4⁺, or CD8⁺ T cells in this region. Such presentation may be qualitatively different from similar processes occurring in other organs, since there is a special propensity for T cells developing in PPs to mediate oral tolerance and IgA B cell development.

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