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*Brief Definitive Report*

# Dendritic Cells Are the Principal Cells in Mouse Spleen Bearing Immunogenic Fragments of Foreign Proteins

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## Summary

We monitored the APC function of cells taken from the spleen and peritoneal cavity of mice that had been given protein antigens via the intravenous or intraperitoneal routes. Using the mAb 33D1 and N418 to negatively and positively select dendritic cells, we obtained evidence that dendritic cells are the main cell type in spleen that carries the protein in a form that is immunogenic for antigen-specific T cells. In vivo pulsed macrophages were not immunogenic and did not appear capable of transferring peptide fragments to dendritic cells.



Before proteins can be recognized by T lymphocytes, most are fragmented into peptides that are bound to MHC products on the surface of APCs. For class II MHC products, peptides can form extracellularly (1) or after endocytosis (2). These events have been studied in tissue culture systems. It is necessary to define pathways of presentation in situ. When antigens are administered to animals, and the dendritic cells are isolated, these APC are carrying the antigen in an immunogenic form. Thus, the in vivo pulsed dendritic cells can stimulate specific T cells in vitro in the absence of additional antigen (3–7). Here we use a variety of approaches for enriching and depleting the trace subset of spleen dendritic cells to assess their importance relative to other APC in carrying immunogen in situ.

## Materials and Methods

*Mice.* CxDF1 (H-$2^d$) and B6.H-2k mice of both sexes were purchased from the Trudeau Institute (Saranac Lake, NY) and used at 6–12 wk of age.

*Antigens.* Mice were pulsed with antigen in vivo by giving 1–4 mg of spermwhale myoglobin, conalbumin, or ovalbumin (Sigma Chemical Co., St. Louis, MO) i.v. or i.p., in 50 or 200 μl PBS, respectively.

*Antigen-specific T Cell Responses.* MHC class II–restricted, myoglobin-specific T cell clones (8) were generously provided by Drs. A. Livingstone and G. Fathman, Stanford University (Stanford, CA). The clones were stimulated with graded doses of in vivo pulsed APC in medium consisting of RPMI-1640 supplemented with 5% FCS, 50 μM 2-ME, and 20 μg/ml gentamicin. DNA synthesis was measured at 48–60 h using 1 μCi [$^3$H]TdR (6.0 Ci/mMol; 4 μCi/ml). Primary populations of myoglobin- and conalbumin-specific T cells were prepared by a new method that reduced the syngeneic MLR that occurs whenever dendritic cells and T cells are cocultured (9). 9–10 d after priming with 100 μg of protein with CFA in front or rear foot pads, the draining lymph nodes were cultured for 10 d in 16-mm wells, at 5 × $10^6$ cells/well in Click's medium (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% mouse serum, 50 μM 2-ME, 2 mM glutamine, and 20 μg/ml gentamicin. At 10 d, the primed cells were enriched on Ficoll-Hypaque columns, and 3 × $10^4$ T cells challenged with graded doses of APC in flat microtest wells. DNA synthesis was measured as above.

*APC.* Spleens were digested with collagenase and separated into low and high density fractions on BSA columns (10). The low density fraction was used, since this had most of the APC function after in vivo antigen pulsing. For negative selection experiments, low density cells were irradiated with 1,000 rad $^{137}$Cs, suspended in mAb and fresh rabbit serum (Complement; Pel-Freeze Biologicals, Rosen, AR), and plated in 16-mm wells for 1 h at 37°C. After gentle pipetting over the surface, adherent cells remained that had been selectively depleted with mAb. The mAbs which are all available from the American Type Culture Collection, Rockville, MD (10) were: 13.4 anti-thy-1, (TIB99, T cells); 33D1 (TIB227, dendritic cells); J11d heat-stable antigen (TIB183, B and some dendritic cells) (10); B21-2 (TIB 229, anti-Ia); and 14.8 anti-B220 (TIB 164, B cells). For positive selection of dendritic cells, low density cells were stained with the hamster mAb N418 followed by biotin rabbit anti-hamster Ig and PE-avidin. N418 identifies the p150/90 leukocyte integrin, most likely murine CD11c, and primarily stains dendritic cells in mouse spleen (11; Crowley, M., K. Inaba, M.D. Witmer-Pack, S. Gezelter, and R.M. Steinman, manuscript submitted for publication). Low density cells were sorted on a FACStar Plus instrument into N418$^+$ and N418$^-$ fractions, the former being 7–10% of low density cells.

The other APC populations that were tested were resident peritoneal cells, enriched for macrophages by adherence to plastic; spleen dendritic cells purified from cultured, adherent low density, populations (10); and cultured epidermal Langerhans cells (12).

## Results

*Adherent Cells with a Low Buoyant Density Are the Main Source of Immunogen in Lymphoid Organs of Mice that Have Been Pulsed*

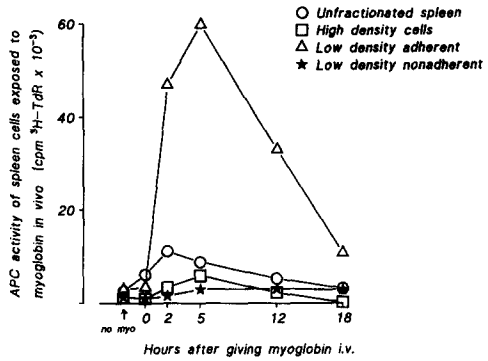


**Figure 1.** Low density spleen adherent cells are enriched for APC activity after an antigen pulse in situ. Groups of four [BALB/C × DBA/2]$F_1$ mice were given 4 mg sperm whale myoglobin i.v. 0–18 h before testing their spleen cells for the capacity to stimulate $10^5$ cells from clone 11.3.7, which is specific for myoglobin plus H-$2^d$. Unfractionated spleen cells were compared with high and low buoyant density fractions at 4 × $10^6$ cells/culture. The low density cells also were separated into adherent and nonadherent fractions. 20% aliquots of the cultures were analyzed for [$^3$H]TdR uptake into DNA. The data are means of triplicates in which standard deviations were <10% and are representative of three similar experiments.

*Intravenously or Intraperitoneally with Foreign Protein.* When spleens were removed from mice that were given myoglobin i.v., the spleen cells could stimulate the myoglobin-reactive, H-$2^d$-restricted, T cell clone 11.3.7 in vitro without any addition of exogenous myoglobin. Stimulation by unfractionated spleen cells was maximal 2 h after administering the protein in vivo and then decreased progressively over an 18-h period (Fig. 1). This APC function was very much enriched in adherent cells with a low buoyant density (Fig. 1), which contain most of the dendritic cells (10, 13). Spleen cells in the high density and nonadherent low density fractions contain other types of APC, such as macrophages and B lymphocytes, but had little or no APC activity even at much higher doses, 1–4 × $10^6$ cells/macroculture.

The observations were repeated with three myoglobin-specific, H-$2^d$-restricted clones (8) (Table 1). For each, strong responses were induced by low density adherent cells from H-$2^d$ mice given myoglobin 2 h beforehand. The APC function likely was MHC-restricted, because in vivo pulsed H-$2^k$ cells provided little (clone 11.3.7) or no (clones 11.12 and 8.2) APC function.

**Table 1.** *APC Activity from Mice Given Myoglobin: Comparison of Different T Cell Clones and MHC Restriction*

| Mice used to provide spleen cells | Proliferation ([$^3$H]TdR uptake) | | |
|---|---|---|---|
| | Clone 11.12.8 | Clone 11.3.7 | Clone 8.2.1 |
| | | *cpm × $10^{-3}$* | |
| H-$2^d$, no myoglobin | 0.7; 1.0 | 5.0; 3.2 | 2.4; 1.3 |
| H-$2^d$, + myoglobin | 14.5; 12.3 | 96.5; 86.3 | 46.3; 39.0 |
| H-$2^k$, no myoglobin | 2.0; 1.7 | 6.5; 4.6 | 2.8; 1.6 |
| H-$2^k$, + myoglobin | 2.7; 2.9 | 15.0; 9.1 | 1.0; 1.0 |

Groups of 4 H-$2^d$ or H-$2^k$ mice were given 4 mg myoglobin i.v. 2 h before they were killed. Low density spleen cells were isolated (10), irradiated, and plated at 5 and 2 × $10^6$ cells in 16-mm tissue culture wells for 1 h. The adherent fraction (~10% of the low density cells and 1% of total spleen) was cultured with $10^5$ T cells from three myoglobin-responsive clones. The data are mean DNA synthesis for duplicate cultures, the two values representing the doses of 5 and 2 × $10^6$ starting low density cells. Background [$^3$H]TdR uptakes in spleen alone, or clone alone, ± myoglobin were 2 × $10^3$ cpm or less and are not subtracted. The experiment was repeated twice with similar results.

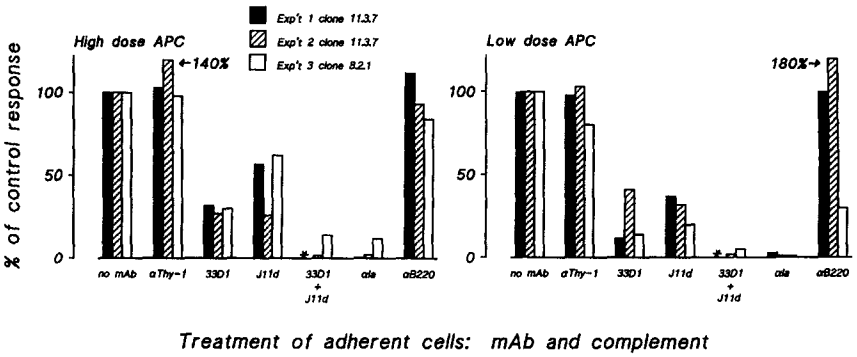


**Figure 2.** The dendritic cell is critical for the APC activity of in vivo pulsed spleen. Groups of three H-$2^d$ mice, which were or were not given 4 mg of myoglobin i.v. 2 h before they were killed, were tested for APC activity. Low density cells were irradiated (1,000 rad) and added to 16-mm wells at 5 or 2 × $10^6$ cells (high and low dose APC) in the presence of the indicated mAb and fresh rabbit serum. After 60 min at 37°C, the nonadherent cells were removed, leaving irradiated adherent spleen cells that had been depleted of leukocytes bearing the respective surface antigens. $10^5$ cloned T cells were added, and DNA synthesis was monitored on the 3rd day. The combination of 33D1 and J11d was not tested in the first experiment (•). Responses in the absence of in vivo pulsing were <500 cpm.

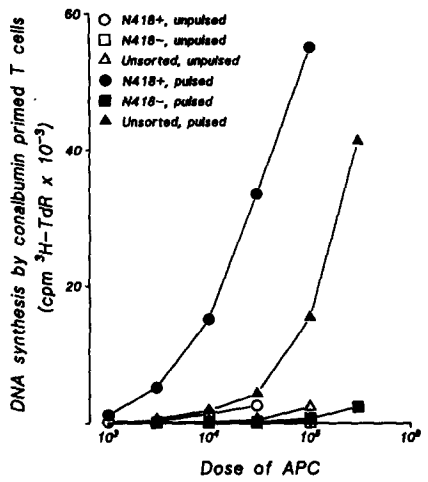


**Figure 3.** Positively selected dendritic cells are enriched in APC activity. Groups of mice were either given or not given 4 mg of conalbumin i.v. 2 h before they were killed. The low density adherent cells from both groups of mice were stained with the hamster mAb N418 (11) and sorted into $N418^+$ and $N418^-$ fractions. The in vivo pulsed APC were then cultured with primary populations of antigen-primed T cells (see Materials and Methods). The data shown are means of triplicate microcultures and are representative of four similar experiments.

In other experiments (not shown), APC activity increased progressively as the dose of myoglobin was increased from 1 to 4 mg per animal. Small amounts of APC activity were detected in thymus and lymph node, but none in the macrophage-rich peritoneal cavity, even when myoglobin was given i.p. In node and thymus, all APC function was in the low density adherent fraction and was difficult to detect unless APC were enriched by this method.

*Negative Selection Approaches Demonstrating that the Main APC is a Dendritic Cell.* Spleen cells from antigen-pulsed mice were irradiated with 900 to 3,000 rad (with similar results) and adhered to plastic in the presence of different mAb and complement (Fig. 2). Removal of T or B cells with Anti-Thy-1 or anti-B220 mAb had no effect on presentation. Removal of $Ia^+$ cells, which kills all types of APC, totally blocked function. A more selective approach was to use 33D1 and/or J11d. Neither mAb killed macrophages, but each killed a fraction of dendritic cells (10) and reduced presentation, particularly when used together.

*Positive Selection of Dendritic Cells with N418 mAb.* Several approaches extended the evidence that dendritic cells were the principal APC in spleen that carried immunogen in vivo. As shown in Fig. 3, the APC activity of in vivo pulsed dendritic cells was evident with (*a*) other proteins (conalbumin in Fig. 3; ovalbumin and rhodamine-conjugated ovalbumin, not shown), (*b*) primary T cell populations rather than clones, and (*c*) positively selected dendritic cells. Positive selection utilized a new hamster mAb N418 which reacts with an epitope on a p150/90 leukocyte integrin that is abundant on dendritic cells but not on most macrophages and lymphocytes (11). N418 does not interfere with APC function (11) and can be used to sort spleen dendritic cells (Crowley, M., et al., submitted for publication). After sorting, $N418^+$ cells were 30–50 times more potent than $N418^-$ cells in stimulating antigen-primed T cells (Fig. 3).

*Mixing Protocols to Rule Out Processing and Peptide Regurgitation by Macrophages.* It remained possible that in situ macrophages were actually processing the antigen and regurgitating peptides that were then presented by dendritic cells. We studied this possibility in vitro by taking advantage of prior results showing that, after dendritic cells are cultured overnight, these APC present peptide fragments but not native protein (14). We confirmed this and proceeded to mix antigen-pulsed H-$2^k$ or H-$2^d$ adherent cells from either spleen (Fig. 4) or peritoneal cavity (not shown) with H-$2^d$ dendritic cells to see if the adherent macrophages would regurgitate peptides for presentation by the added H-$2^d$ dendritic cells. The addition of macrophages (3–30 × $10^4$/well) did not allow the cultured H-$2^d$ dendritic cells to stimulate the

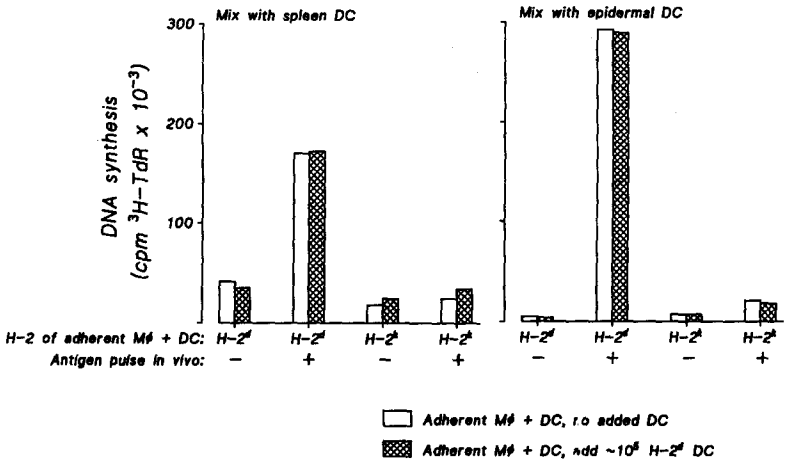


**Figure 4.** Antigen-pulsed macrophages do not regurgitate peptides for presentation by dendritic cells. Groups of three to five CxD2 [H-$2^d$] or B6.H-$2^k$ mice were not treated or were given 2 mg of myoglobin i.p. 2 h before they were killed. Low density adherent cells were prepared from collagenase digested spleens (~$10^5$ adherent cells/16-mm well) (10, 13). The adherent cells were cultured with 2 × $10^5$ myoglobin-specific clone 11.3.7 T cells (which responds much better to antigen in the context of I-$E^d$ than I-$E^k$, Table 1). To some of the cultures we added 8.5 × $10^4$ cultured H-$2^d$ epidermal Langerhans cells or 1.2 × $10^5$ H-$2^d$ spleen dendritic cells. This allowed us to test if immunogenic peptides were released from the adherent population, since after overnight culture, these dendritic cell populations do not present native myoglobin but can present peptide fragments (14). The data are shown for duplicate wells, and are representative of three similar experiments. Uptakes by the APC only, or the clone only, were <0.5 × $10^3$ cpm. A similar failure to detect peptide regurgitation was observed when myoglobin was applied continuously at 0.1 mg/ml or when adherent peritoneal cells, primarily macrophages, were tested from mice pulsed with myoglobin i.p.

T cell clones, even if myoglobin was added continuously at 100 μg/ml (not shown), indicating that peptides are not transferred from macrophages to dendritic cells in vitro.

## Discussion

These data extend previous work (3–7) by showing that in situ the dendritic cell is a major source of immunogen (Figs. 2, 3) for antigen-specific T cells. Immunogen that is acquired by dendritic cells may be generated by extracellular proteolysis (1) or by regurgitation of peptides from other cells. We were not able to find evidence for such pathways in vitro, however. When dendritic cells were cultured together with macrophages, the latter did not release material that could be presented by dendritic cells (Fig. 4). Therefore, we propose that endocytosis and processing are carried out independently by dendritic cells. If so, the amount of protein that needs to be endocytosed is relatively small, since dendritic cells accumulate only small amounts of an endocytic load in vivo (13). Perhaps endocytosis in dendritic cells is exclusively devoted to antigen presentation, since these APC seem to function primarily to initiate cell-mediated immunity rather than as effector cells for clearing antigen. In contrast, antigen uptake is readily visualized and quantitated in macrophages (13, 15), but much of the observed uptake functions to clear and destroy antigen.

Our findings could be explained if in vivo antigen-pulsed dendritic cells (*a*) process or (*b*) retain antigen more efficiently, (*c*) provide stronger "second" signals, or (*d*) are positioned in areas where antigen accesses lymphoid tissue, for which evidence was just obtained (11). Further understanding awaits better methods for quantitating MHC-peptide complexes on living APC. For the time being, the isolation of dendritic cells from immunologically stimulated hosts may provide a way to isolate APC carrying relevant epitopes for T cells in situ.

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