

# Dendritic Cell Progenitors Phagocytose Particulates, Including *Bacillus Calmette-Guerin* Organisms, and Sensitize Mice to Mycobacterial Antigens In Vivo

By Kayo Inaba,\* Muneo Inaba,† Makoto Naito,§  
and Ralph M. Steinman||

From the \*Department of Zoology, Faculty of Science, Kyoto University, Kyoto 606; the †First Department of Pathology, Kansai Medical University, Osaka 570; the §Second Department of Pathology, Niigata University School of Medicine, Niigata 951, Japan; and the ||Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

## Summary

Dendritic cells, while effective in sensitizing T cells to several different antigens, show little or no phagocytic activity. To the extent that endocytosis is required for antigen processing and presentation, it is not evident how dendritic cells would present particle-associated peptides. Evidence has now been obtained showing that progenitors to dendritic cells can internalize particles, including *Bacillus Calmette-Guerin* (BCG) mycobacteria. The particulates are applied for 20 h to bone marrow cultures that have been stimulated with granulocyte/macrophage colony-stimulating factor (GM-CSF) to induce aggregates of growing dendritic cells. Cells within these aggregates are clearly phagocytic. If the developing cultures are exposed to particles, washed, and "chased" for 2 d, the number of major histocompatibility complex class II-rich dendritic cells increases substantially and at least 50% contain internalized mycobacteria or latex particles. The mycobacteria-laden, newly developed dendritic cells are much more potent in presenting antigens to primed T cells than corresponding cultures of mature dendritic cells that are exposed to a pulse of organisms. A similar situation exists when the BCG-charged dendritic cells are injected into the footpad or blood stream of naive mice. Those dendritic cells that have phagocytosed organisms induce the strongest T cell responses to mycobacterial antigens in draining lymph node and spleen. The administration of antigens to GM-CSF-induced, developing dendritic cells (by increasing both antigen uptake and cell numbers) will facilitate the use of these antigen-presenting cells for active immunization in situ.

There is increased interest in the use of constituent proteins and peptides to modulate T cell responses to complex microbial and cellular antigens in situ. Typically, artificial adjuvants are required. Several antigens are known to be immunogenic when administered in association with dendritic cells but in the absence of additional adjuvants (1). The immunogenicity of dendritic cells in situ is apparent with contact allergens (2), transplantation antigens (3–6), and more recently foreign proteins (7–9). The dendritic cells are serving directly as APCs in situ, because the T cells that are primed are restricted to recognize antigens presented by the MHC of the immunizing dendritic cells rather than host APCs (7, 9). These observations, when coupled with data that dendritic cells are efficient at capturing protein antigens in an immunogenic form in situ (10, 11, 12), make it apparent that these dendritic cells are "nature's adjuvant."

Dendritic cells offer the potential of processing complex antigens into those peptides that would be presented by self-

MHC products. A possible restriction to this immunizing potential relates to the fact that in most species and tissues, dendritic cells are not actively phagocytic. Since endocytosis is an important means whereby antigens are processed to form the peptides that are presented on MHC class II products (13–15), it is not apparent how dendritic cells would present particle-derived peptides. The paradox is heightened by recent data that phagocytic macrophages do not appear to regurgitate peptides for presentation by dendritic cells (12).

Here we describe an apparent solution to this issue. We will show that dendritic cells can internalize particulates during an early stage in their development from proliferating progenitors. Prior work established that the stimulation of bone marrow suspensions with GM-CSF leads to the production of clusters of proliferating dendritic cell precursors (16). The cells that pulse label with [<sup>3</sup>H]thymidine in the clusters lack many of the characteristic markers of dendritic cells, e.g., stellate shape and antigenic features like NLDC-145 antigen and

high levels of MHC class II. In pulse-chase experiments, [<sup>3</sup>H]thymidine-labeled progeny with all the features of dendritic cells are released. We have found that cells within the aggregate also are phagocytic, and that in analogous pulse-chase protocols, the progeny dendritic cells are clearly labeled with the phagocytic meal. When the particles are *Bacillus Calmette-Guerin* (BCG)<sup>1</sup> organisms, mycobacterial antigens are presented in a potent manner to T cells *in vitro* and *in situ*.

## Materials and Methods

**Mice.** (BALB/c × DBA/2)F<sub>1</sub>, (C57BL/6 × DBA/2)F<sub>1</sub>, and BALB/c male and female mice were purchased from the Trudeau Institute (Saranac Lake, NY) and Japan SLC (Hamamatsu, Shizuoka) and used at 6–10 wk of age.

**Bone Marrow Culture.** As described (16), bone marrow was flushed from the femurs and tibias, depleted of red cells with 0.83% ammonium chloride, and nonlymphoid Ia<sup>-</sup> cells were cultured in 24-well plates (Nunc, Naperville, IL; and 25820; Corning Science Products, Rochester, NY) at 10<sup>6</sup> cells per well in 1 ml of RPMI 1640 supplemented with 5% FCS, 20 μg/ml gentamicin, and 1,000 U/ml recombinant murine GM-CSF (9.7 × 10<sup>7</sup> U/mg; Kirin Brewery, Maebashi, Gumma, Japan). At 2 d, 0.75 ml of medium and most of the nonadherent cells were removed and replaced with fresh medium. This was repeated at days 4–5, thereby removing most of the developing granulocytes and leaving behind clusters of proliferating dendritic cells adherent to a stroma that included scattered macrophages. The culture medium was then supplemented with particulates, and phagocytosis was allowed to proceed for 20–24 h, usually on days 5–6. At this point the cultures were rinsed free of loose cells and particles, and the cells were analyzed immediately for particle uptake. Alternatively, cells in the washed cultures were dislodged and 3–4 × 10<sup>6</sup> cells were transferred to a 60-mm petri dish for 1- or 2-d “chase” period in particle-free, fresh, GM-CSF-supplemented medium. Class II-rich, mature dendritic cells developed during the chase as described (16), and these were isolated by cell sorting (below). To compare the phagocytic activity of developing and mature dendritic cells, particles were also administered to 7–8-d bone marrow cultures that are rich in single nonproliferating mature dendritic cells.

**Particulates.** BCG mycobacteria at 1.5–2.5 × 10<sup>8</sup> CFU/ml (Trudeau Institute) and 4 × 10<sup>8</sup> CFU/ml (Kyowa Pharmaceutical Industries, Tokyo, Japan) were administered at ~10<sup>7</sup> live BCG per 16-mm diameter well. Uptake was assessed following an “acid-fast” stain using an auramine-rhodamine procedure (17) that is more sensitive than Ziehl Neelsen and facilitates organism counts. Colloidal carbon (Pellikan Ink, Hannover, Germany) was added at 1:2,000 dilution. The carbon was identified as a black granular stain in specimens stained with Diff-Quik® (Baxter Healthcare Corp., Miami, FL). Latex particles (0.5% [vol/vol], 2-μm diameter; Seradyn, Indianapolis, IN) were applied to 1 ml of culture in a 16-mm-diameter well at 50 μl/well.

**Isolation of Mature Dendritic Cells by Cell Sorting.** As noted before (16), the dendritic cells that are produced in GM-CSF-stimulated bone marrow cultures express very high levels of surface MHC class II products (mAbs B21-2; TIB 229, and M5/114; TIB 120 from the American Type Culture Collection [ATCC], Rockville, MD) as well as moderate levels of a dendritic cell-restricted antigen recognized by mAb NLDC-145 (18). Immediately after the pulse with

BCG, or after an additional 2 d of “chase” culture, the cells were stained with biotin B21-2 and FITC-streptavidin (Tago, Burlingame, CA). Class II-rich cells then were sorted (FACStar Plus®; Becton Dickinson Immunocytometry Systems, San Jose, CA) and cytospun onto glass slides (Shandon Southern Instruments Inc., Sewickley, PA). Sorted cells were stained with Diff-Quick® (Baxter Healthcare Corp.), which outlines the stellate shape of dendritic cells in cytopins and allows enumeration of profiles containing perinuclear depots of internalized colloidal carbon or latex spheres. To visualize BCG, the cytopins were fixed in absolute acetone for 10 min at room temperature and stained with M5/114 anti-class II, NLDC-145 anti-dendritic cell (18), or RA3-6B2 anti-B220 or anti-B cell (the latter as a control) followed by POX-conjugated mouse anti-rat Ig (Boehringer Mannheim Biochemicals, Indianapolis, IN) and diaminobenzidine tetraHCl (Polyscience Inc., Warrington, PA). The preparations were then double labeled for acid-fast bacilli with auramine rhodamine (17). Virtually all the cells in the preparation were rich in NLDC-145 and MHC class II products. The numbers of BCG bacilli in at least 400 cells were enumerated.

**Electron Microscopy (EM).** To prove that cell-associated BCG were all internalized, the dendritic cells produced in pulse-chase protocols (above) were fixed in 2.5% glutaraldehyde and processed for EM as described (16).

**Antigen Presentation *In Vitro*.** Mice were primed with CFA (Sigma Chemical Co., St. Louis, MO), 25 μl in the fore and rear paws, or, as a control, mycobacteria-free IFA. 1–2 wk later, the draining lymph nodes were dissociated into a single-cell suspension and depleted of APCs with mAbs to MHC class II, B220, and heat-stable antigens (M5/114 anti-Ia, RA3-6B2 anti-B220, and J11d anti-HSA; TIB 120, 146, and 183 from the ATCC, respectively) and rabbit complement. 3 × 10<sup>5</sup> of these APC-depleted, primed T cells were cultured in 96-well flat-bottomed microtest wells (Nunc; or 25860; Corning Science Products) in Click’s medium (Irvine Scientific, Santa Ana, CA) supplemented with 0.5% normal mouse serum, 20 μg/ml gentamicin, 50 μM 2-ME, 2 mM glutamine, and 1 mM sodium pyruvate (Gibco Laboratories, Grand Island, NY). Graded doses of BCG-pulsed, bone marrow or spleen APCs were added (see Results). 1 μCi of [<sup>3</sup>H]thymidine (20 Ci/mmol, 4 μCi/ml; New England Nuclear, Boston, MA; or 15 Ci/mmol, 4 μCi/ml; American Radiolabeled Chemicals Inc., St. Louis, MO) was added to monitor DNA synthesis at times indicated in the legends. Data shown are means of triplicates in which SDs were <15% of the mean.

**Antigen Presentation *In Vivo*.** APCs that had been pulsed with antigen *in vitro* were administered *in vivo* to unprimed (C×D2)F<sub>1</sub> mice. To prime T cells in draining lymph node, 2 × 10<sup>5</sup> dendritic cells were injected into the paws and node cell suspensions were prepared 5 d later (7). To prime T cells in spleen, 10<sup>6</sup> cells were injected intravenously and splenocytes were prepared 5 or 10 d later. To measure T cell priming, bulk lymph node or spleen cells were cultured as above and challenged with graded doses of protein antigens, either purified protein derivative (PPD; Statenserum Institute, Copenhagen, Denmark, or Dr. Ichiro Toida, Research Institute for BCG in Japan, Kiyose, Tokyo), or BSA, and [<sup>3</sup>H]thymidine uptake was measured at 48–64 h. To characterize the proliferating cells, the populations were treated with antibodies and complement before measuring [<sup>3</sup>H]thymidine uptake.

## Results

**Phagocytosis of Latex Particles within Clusters of Developing Dendritic Cells: Pulse and Pulse-Chase Protocols.** When mouse

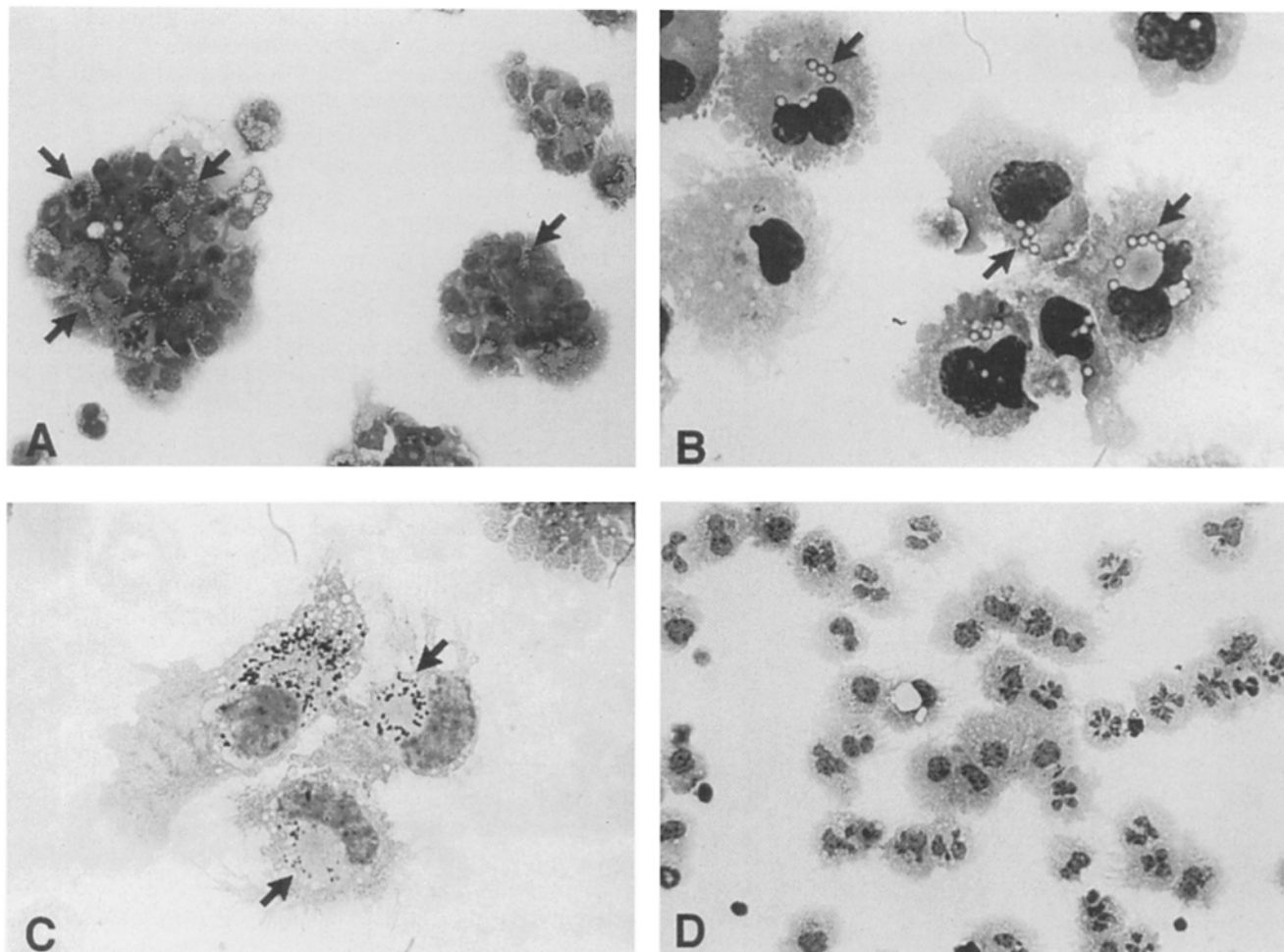
<sup>1</sup> Abbreviations used in this paper: BCG, *Bacillus Calmette-Guerin*; PPD, purified protein derivative.

blood or bone marrow is stimulated with GM-CSF, proliferating cell aggregates appear, and these give rise to large numbers of typical immunostimulatory dendritic cells (16, 19). In bone marrow, which was used for the experiments described below, the proliferating aggregates are best identified by washing away the majority of nonadherent granulocytes that are also induced by GM-CSF in the cultures (16). At days 5–6, the time point when the aggregates were first sizable (5–10 cells wide), we applied different particles over a 20–22-h period.

After administration of 2- $\mu$ m latex spheres, heavy labeling was noted in scattered macrophages on the monolayer. In addition, some clear labeling occurred within the developing dendritic cell aggregates (Fig. 1 A). Aggregates that had been exposed to particles were recultured an additional 2 d. During this time, large numbers of cells were released into suspension. These primarily were mature dendritic cells with char-

acteristic stellate shapes and high levels of MHC class II and NLDC-145 antigens (16). When the released cells were examined by light microscopy, many contained latex spheres that were often around a clear perinuclear zone or centrosphere (Fig. 1 B). We also studied colloidal carbon uptake in a similar manner. When aggregates were pulsed with colloid and mature dendritic cells were allowed to form during a chase period, some of the released cells had a centrosphere with small but clear-cut carbon deposits (Fig. 1 C). In contrast, when latex or carbon was offered to mature dendritic cells, little uptake took place (Fig. 1 D).

*BCG Uptake by Developing Dendritic Cells: Acid-fast Stains.* The same protocols were repeated, but now preparations with live BCG mycobacteria were administered as the phagocytic meal. Cell-associated bacilli were visualized by a sensitive fluorescent acid-fast stain. After the 20-h pulse, the developing dendritic cell aggregates were again strongly labeled. To iso-



**Figure 1.** Diff-Quick stains of developing dendritic cells that have been exposed to Latex and carbon. (A) An aggregate of developing dendritic cells cytospun after a 20-h exposure to 2- $\mu$ m latex spheres. Many cells in the aggregate are labeled with the uniform latex particles (arrows).  $\times 270$ . (B) Same as A, but the cultures were chased for 1 d to allow the production of mature single dendritic cells. Many of the released dendritic cells contain the uniform and lucent latex spheres (arrows).  $\times 675$ . (C) Same as A and B, but the aggregates were pulsed with colloidal carbon and then chased for 1 d in carbon-free medium. The centrospheres of some of the mature dendritic cells that release from the aggregate contain small but clear-cut endocytic granules of black, indigestible phagocytic tracer (arrows).  $\times 675$ . (D) Mature dendritic cells were exposed to carbon after they had been produced from proliferating aggregates. Few if any carbon deposits are evident.  $\times 270$ .

late the more mature dendritic cells from the cultures, we resuspended the cells and sorted those with high levels of MHC class II products. Immediately after the BCG pulse, ~20% of the sorted cells contained acid-fast bacilli (Table 1). It was difficult to separate the majority of MHC class II-weak cells because of excessive stickiness during cell sorting.

Companion cell cultures were then studied after 2 d of a chase culture. Because many mature dendritic cells formed during the chase period, the number of Ia-rich progeny had increased fourfold (Table 1). About 50% of these progeny contained BCG, usually in a perinuclear location (Table 1 and Fig. 2). Double labeling experiments verified that the cells containing acid-fast bacilli expressed MHC class II and the dendritic cell-restricted NLDC-145 antigen (Fig. 2). Because the total number of MHC class II- and NLDC-145-positive cells had also increased fourfold in just 2 d, it is likely these BCG-laden dendritic cells were derived from less mature but phagocytic progenitors in the aggregates.

**Electron Microscopy of BCG-pulsed APCs.** The perinuclear location of the cell-associated particles by light microscopy indicated that organisms had been internalized. The matter was verified by electron microscopy. Most of BCG organisms were identified intracellularly. All cell-associated BCG were identified within intracellular vacuoles close to the Golgi complex (Fig. 3, A and B). Typically only one or two bacilli could be identified within any one section through the dendritic

cell centrosphere. It appeared that a phagosomal membrane closely approximated most bacilli (Fig. 3 C and D).

**Presentation In Vitro of Mycobacterial Antigens to Primed T Cells.** To test the presenting function of dendritic cells that had been pulsed or pulse chased with BCG organisms, we first prepared antigen-responsive T cells from the draining lymph nodes of mice that had been injected with CFA that contains heat-killed mycobacteria or with IFA as control (see Methods). When dendritic cells were added to IFA-primed T cells, a syngeneic mixed leukocyte reaction was observed. This was comparable whether or not the APCs had been exposed to BCG (Fig. 4, right). However, when dendritic cells had been pulsed with BCG and were added to CFA-primed T cells, strong proliferative responses were induced (Fig. 4, left). If dendritic cells were tested immediately after the 1-d pulse, or after an additional 2-d chase period, the chased population was much more potent (Fig. 4, left, compare diamonds with inverted triangles). As few as 100 BCG pulse-chased dendritic cells elicited sizable T cell responses in vitro (Fig. 4 left, diamonds). The BCG-pulse-chased populations also were 5–10 times more potent in inducing responsiveness to mycobacterial antigen than mature dendritic cells freshly exposed to either PPD or BCG (Fig. 4, left, compare diamonds with circles and triangles). Therefore, it appeared that the extent of phagocytosis correlated with the efficacy of presentation, as the pulse-chased populations were the most active APCs and contained the most intracellular BCG (Table 1).

**Presentation In Vivo of Mycobacterial Antigens to Unsensitized Mice.** Comparable populations of BCG-pulsed, and BCG-pulsed and chased, APCs were tested for the capacity to present mycobacterial antigens to unprimed mice. After injection into the footpads, strong responsiveness to PPD was observed (Fig. 5). Again, the dendritic cells were the most potent if tested after a 2-d chase (Fig. 5; compare open diamonds with open triangles), and this chase period greatly increased the total yield of dendritic cells (16).

To test if the increased antigen-presenting function of BCG pulse-chased dendritic cells was related to the increased number of APCs carrying BCG, the primed populations were also tested for responsiveness to BSA, since the dendritic cells had been grown in the presence of FCS. All the dendritic cell populations, regardless of the details of the exposure to BCG, primed mice similarly to BSA (Fig. 5, filled symbols). This indicates that each population was comparably efficient in immunizing to a soluble protein, whereas the dendritic cells that had phagocytosed BCG were more effective in eliciting responses to mycobacterial antigens.

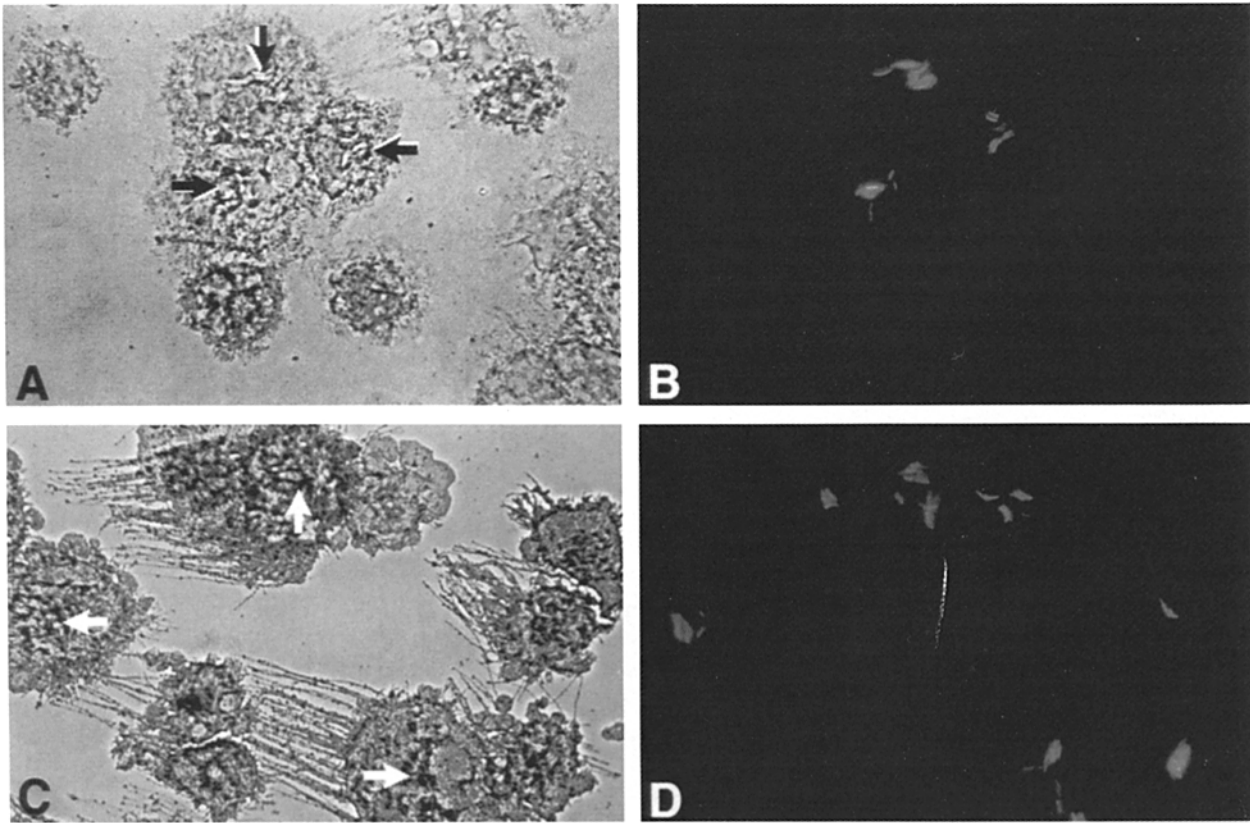
The surface markers of the primed cells were tested by antibody- and complement-mediated lysis of the populations before measuring [<sup>3</sup>H]thymidine uptake (data not shown). The proliferating cells were Thy-1, but negative for MHC class II, heat-stable antigen, and B220. Anti-CD4 hybridoma culture supernatant blocked proliferation >85%, i.e., the primed cells were Th cells.

Priming was also observed when spleen T cells were tested after an intravenous infusion of BCG-pulsed and BCG pulse-chased dendritic cells (Fig. 6). The cells were more

**Table 1.** Frequency of Dendritic Cells with Phagocytosed BCG Organisms in GM-CSF-stimulated Mouse Bone Marrow Cultures

Exp.	BCG exposure	No. of cells counted	Phagocytic cells	BCG/DC
			%	
1	Days 5–6 pulse	469	18.1	2.6
		498	18.5	2.5
2	Days 5–6 pulse	444	22.5	3.0
		463	22.2	2.9
	Pulse, 2-d chase	564	57.1	3.8
		579	57.0	3.2
3	Days 5–6 pulse	440	21.8	2.1
		623	22.8	2.9
	Pulse, 2-d chase	487	50.3	2.9
		511	58.7	4.0

Quantitative data of dendritic cells containing BCG. Mouse bone marrow cultures were stimulated in 16-mm wells for 5 d with GM-CSF, washed, and exposed to BCG organisms for 20 h. The cultures were washed again and either examined immediately, or pooled and transferred to a 60-mm dish for an additional 2-d chase culture. The dendritic cells in the cultures were selected as Ia-rich cells using a cell sorter and then spun onto glass slides for staining for acid-fast bacilli. During the chase period, the percentage of Ia-rich cells in the cultures increased 2–2.5-fold, and the total number of cells increased twofold, so that the number of Ia-rich cells increased four to five times.



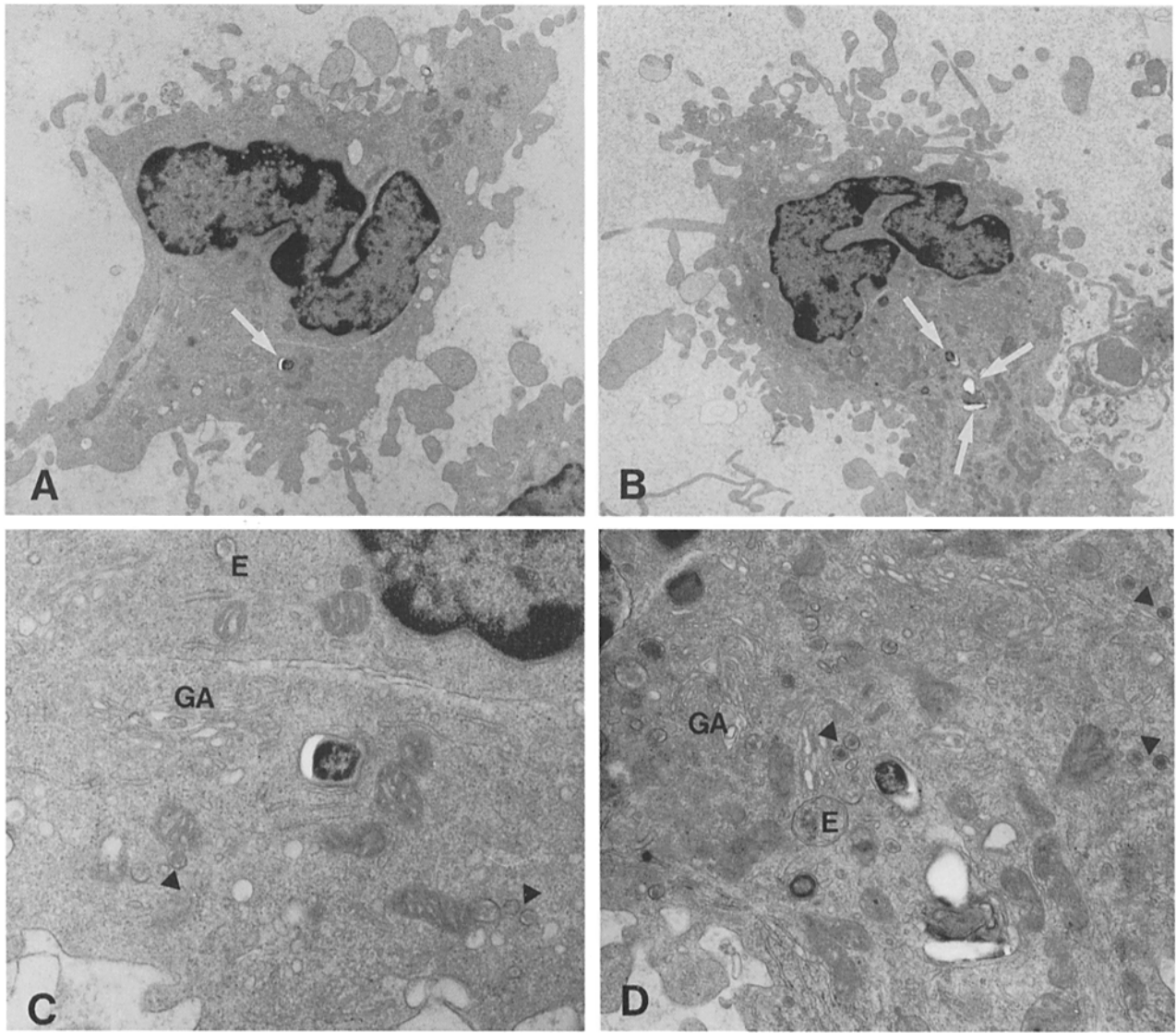
**Figure 2.** Uptake of BCG into developing dendritic cells using two-color labels for acid-fast bacilli and dendritic cell antigens. Clusters of developing dendritic cells (6-d marrow cultures induced with GM-CSF) were exposed for 20 h to BCG. The monolayers were washed and chased in medium with GM-CSF for 2 d. The cells were dissociated, labeled with FITC-anti-I-A mAb, and the class II-rich cells were isolated by cell sorting (most of the cells in the culture are class II rich) as shown previously (16). The sorted cells were cytopun, stained with auramine-rhodamine to visualize the cell-associated BCG (B and D), and double labeled with a different mAb and immunoperoxidase (A and C). Arrows on the left indicate the location of the bacilli. The peroxidase label for anti-class II (I-A and I-E, M5/114) outlines the dendritic cell processes (C) much better than the label for the dendritic cell-restricted NLDC-145 antigen (A). The brown reaction product in the color version of A corresponds to the dense black deposits in the black and white version that is shown.  $\times 600$ .

responsive at 5 vs. 10 d after injection (compare Fig. 6, A with C). Again dendritic cells that had been cultured ("chased") for 2 d after exposure to BCG were the most potent (Fig. 6, compare *filled diamonds* with *filled triangles*), but all populations primed the spleen cells similarly to BSA (Fig. 6 B). We conclude that dendritic cell progenitors capture and retain mycobacterial antigens in a manner that is highly immunogenic *in vivo*.

### Discussion

Particle uptake is a specialized activity of mononuclear and polymorphonuclear phagocytes. Dead cells, immune complexes, and microorganisms all are avidly internalized. After fusion with hydrolase-rich lysosomes, the ingested particles are degraded (20, 21). This degradation must be at the level of permeable amino acids (22, 23) and saccharides, otherwise the vacuolar apparatus would swell with indigestible materials (24, 25). Such clearance and digestive functions of phagocytes contribute to wound healing, tissue remodeling, and host defense.

Another consequence of endocytosis, the processing of antigens by APCs differs in many respects from the scavenging function of phagocytosis. (a) Processing required the generation of peptides at least 8–18 amino acids in length (26, 27), while scavenging entails digestion to amino acids (22, 23). (b) Presentation requires the binding of peptides to MHC class II products, possibly preferentially to newly synthesized class II (28, 29), whereas scavenging does not require MHC products. (c) Antigen presentation can function at a low capacity, since only a few hundred molecules of ligand need to be generated for successful stimulation of certain T-T hybrids (30, 31) and most likely primary T cell populations (32). During scavenging, phagocytes readily clear and destroy hundreds of thousands of protein molecules each hour (23). (d) Antigen presentation is best carried out by cells that are rich in MHC class II but show little phagocytic activity and few lysosomes, i.e., dendritic cells and B cells, whereas phagocytes (macrophages and neutrophils) often have low levels of class II and abundant lysosomes. These observations, together with the identification of antigenic specializations within the endocytic system of dendritic cells and B cells (33, 34), have



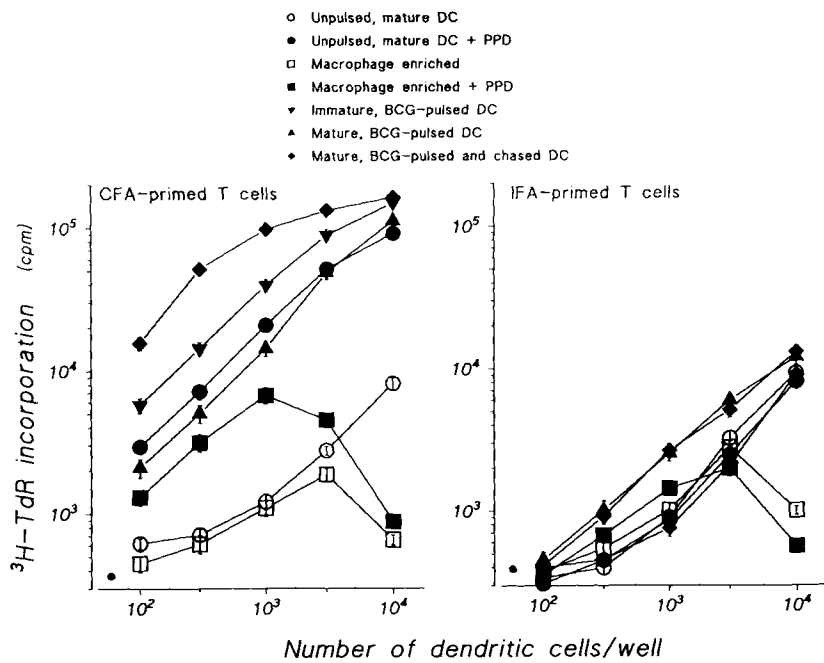
**Figure 3.** Electron microscopy of BCG in dendritic cells. As in Fig. 2, BCG was added to GM-CSF-stimulated 6-d bone marrow cultures for 1 d. After washing and an additional 2 d of culture, the released cells were processed for electron microscopy. (A and B) Low-power views to show the typical dendritic cells with numerous processes and a few phagocytosed BCG (white arrows).  $\times 5,400$  (A);  $\times 3,900$  (B). (C and D) Higher power views to show phagosomal membranes against the BCG, as well as organelles of the dendritic cell centrosphere, including endocytic vacuoles (E), Golgi apparatus (GA), and small vesicles with a dense core ( $\blacktriangle$ ).  $\times 20,000$  (C);  $\times 15,000$  (D).

led to the suggestion that the machinery required for antigen presentation may differ from that required for scavenging, both quantitatively and qualitatively (7). Nevertheless, how do APCs that are not phagocytic (B cells and dendritic cells) present peptides derived from particulates?

In the case of dendritic cells, there have been indications that these APCs are at some point capable of phagocytic activity. Pugh et al. (35) noted Feulgen-stained inclusions in some afferent lymph dendritic cells and suggested that phagocytosis of other cells had taken place before entry into the lymph (35). Fossum and Rolstad (36) noted phagocytic inclusions in the interdigitating dendritic cells of the T cell areas in mice that were rejecting allogeneic leukocytes. Reis

e Sousa et al. (37) found that freshly isolated epidermal Langerhans cells, which are immature but nonproliferating dendritic cells, can internalize small amounts of certain particulates. Here we show that clear-cut phagocytosis occurs when particles are administered to cultures of proliferating dendritic cells. If particles are fed to progenitor populations, phagocytic activity is expanded both qualitatively, i.e., many dendritic cell progeny carry a particulate meal, and quantitatively, i.e., the numbers of such cells are greatly increased.

The number of particles ingested by developing dendritic cells is small relative to typical phagocytes, and the rate of uptake is slow. For example, after a 4-h pulse there is relatively little labeling of dendritic cell precursors in the de-

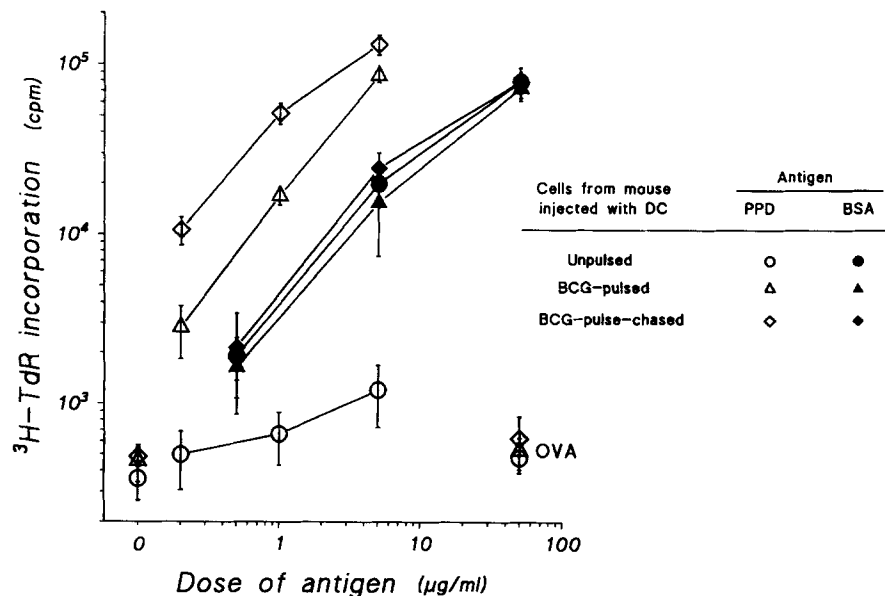


**Figure 4.** Antigen presentation to CFA-primed/IFA-primed T cells. T cells were purified from lymph nodes that drain paws that had been primed with CFA or IFA. The different APCs are listed. Mature dendritic cells are day 8 bone marrow cultures, and immature dendritic cells are from day 5-6 cultures.

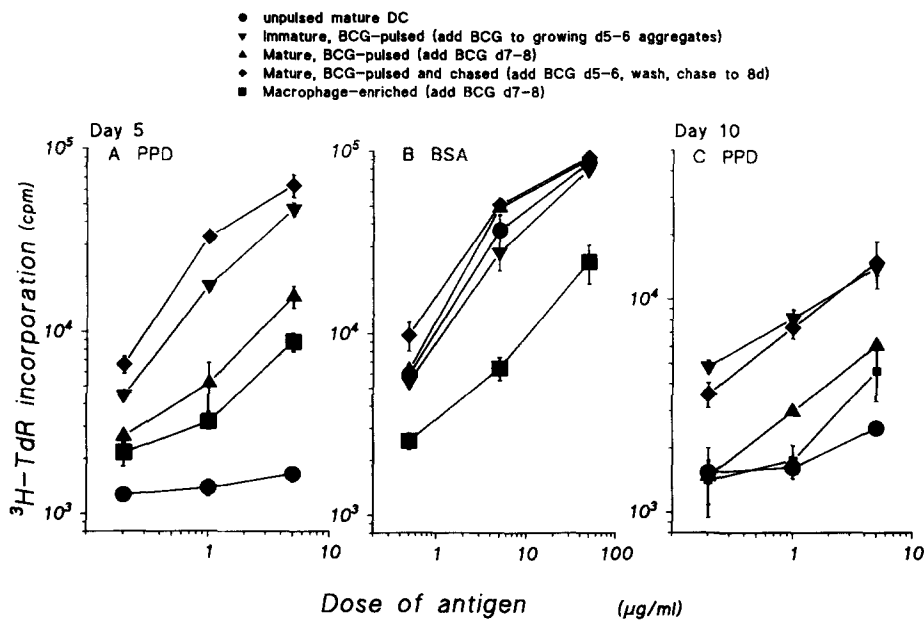
veloping aggregates, while there is active uptake by macrophages on the monolayer. Nonetheless, the administration of particulate meals (latex and BCG) to developing cultures leads to clear-cut phagocytosis by these specialized APCs.

Some sense of the immunogenicity of the ingested particles was obtained with BCG mycobacteria (Figs. 4-6). In any inoculum of the BCG vaccine, there are live bacilli (~50% of the bacilli act as CFU), dead bacilli, and probably a number of mycobacterial proteins. We suspect that the phagocytosed pool of BCG is being presented because of ob-

servations comparing the presentation of mycobacterial antigens with BSA, a component of the serum in which the dendritic cells are grown. Whereas dendritic cells that are pulsed and chased with BCG are the most effective APCs for mycobacteria (Figs. 5 and 6, diamonds) and contain the most particles, all the APC populations were comparable in presenting BSA. This implies that BCG particle uptake accounts for the bulk of the mycobacterial priming. One can now undertake a study wherein dendritic cells are evaluated for their capacity to induce host resistance to mycobacteria



**Figure 5.** Antigen presentation to naive node T cells in situ. Growing cultures of bone marrow dendritic cells were pulsed with BCG at days 5-6, and used immediately or after a 2-d chase culture. The populations were injected into the paws of naive mice without artificial adjuvants. 5 d later the draining lymph nodes were taken and stimulated in vitro with graded doses of PPD and BSA (the dendritic cells had been grown with FCS), with the BSA serving as a nonparticulate antigen. Data are means and SDs for groups of five mice, each studied separately. Control lymph nodes did not respond to PPD or to BSA (<2,000 cpm).



**Figure 6.** Antigen presentation to naive spleen cells in situ. Growing cultures of bone marrow dendritic cells were pulsed with BCG at days 5-6 (immature), at days 7-8 (mature), or at days 5-6 followed by a 2-d chase.  $10^6$  cells were injected intravenously into groups of mice. 5 or 10 d later, the spleen cells were cultured in vitro with graded doses of PPD or BSA as antigen. Since the dendritic cells were cultured in FCS, the use of BSA serves as control to ensure that all dendritic cell populations were comparably immunogenic in vitro. Unprimed spleen did not respond to either BSA or PPD.

infection, a matter of some import given the need to develop better vaccination and treatment protocols for tuberculosis, including the drug-resistant variety.

In effect, the pulse and chase protocol that we have used to charge developing dendritic cells with organisms in the BCG vaccine allows the two broad components of immunostimulation to take place sequentially. These components are: (a) antigen capture and presentation, here the capture of particulates, by immature dendritic cells; and (b) development of potent accessory or immunostimulatory functions during the chase period. The situation is comparable to that seen in the handling of soluble proteins (29, 38) and particles (37) by epidermal Langerhans cells. Each of the two broad components of APC function entails many subcomponents. For example, immature dendritic cells not only are more phagocytic but display other features needed for antigen presentation such as active biosynthesis of abundant MHC class II molecules and invariant chain (29, 39) and numerous acidic endocytic vacuoles (40).

The capacity to charge APCs with antigens in pulse-chase protocols may be a special feature of dendritic cells. Prior studies with macrophages and B cells had suggested that T cell epitopes are short lived (41). The results reported here and elsewhere (7, 29, 32) indicate that immunogenic peptides can be long lived on dendritic cells at least 2 d before injection into mice. This retention capacity should enable dendritic cells to migrate and sensitize T cells in draining lymphoid tissues over a several-day period (7-9).

In this paper bone marrow was used as a ready source of GM-CSF-responsive, proliferating dendritic cell precursors (16). Similar progenitors are found in many other tissues (Inaba

et al., manuscript in preparation) and in the blood itself (19). If progenitors are widely distributed, dendritic cells may acquire particulates whenever and wherever their proliferation and/or maturation is induced. The host may thereby generate in vivo sizable numbers of potent APCs that have successfully processed particulate antigens in that locale.

In the case of BCG, the bulk of the primed cells are  $CD4^+$  T cells, most likely because the antigenic load is handled by the endocytic pathway and MHC class II products (15). An important feature of dendritic cells is the capacity to efficiently present microbial and other antigens on both class I and II products. In the case of influenza, it has been found that the class I pathway for inducing  $CD8^+$  CTL requires adequate delivery of antigen (infectious virus) into the cytoplasm, whereas the purely endocytic pathway delivers noninfectious virions for presentation to  $CD4^+$  helpers (42). Developing dendritic cell cultures provide an opportunity for charging MHC class I products with peptide, since cell proliferation allows various methods of gene insertion (as with retroviral vectors) to be applied.

By using developing dendritic cells to charge MHC class I and/or II products, several desirable components of T cell modulation in situ can be achieved. One brings about antigen uptake and presentation with immature progenitors, allows the APC to tailor the peptides that are appropriate for an individual's MHC products, and increases the numbers of specialized stimulatory APCs. These properties of dendritic cell progenitor populations meet many of the demands for using cells as vehicles for active immunization and immunotherapy in situ.



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Address correspondence to Kayo Inaba, Department of Zoology, Faculty of Science, Kyoto University, Kitashirakawa-Oiwakecho, Sakyo-Ku, Kyoto 606, Japan.

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