Histochem Cell Biol (2003) 120:465–473
DOI 10.1007/s00418-003-0590-8

ORIGINAL PAPER

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Specific localization of epidermal-type fatty acid binding protein in dendritic cells of splenic white pulp

Accepted: 23 October 2003 / Published online: 12 November 2003
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Abstract Dendritic cells in the splenic white pulp of mice were intensely immunoreactive for epidermal-type fatty acid binding protein (E-FABP). This specific immunostaining revealed a clear difference in morphology between the dendritic cells in the periarterial lymphoid sheath (PALS) and follicular dendritic cells in the follicles in terms of cell sizes and process branching. No immunoreactivity was detected in dendritic cells in the marginal zones and the red pulp, although endothelial cells of almost all capillaries in the red pulp were immunoreactive for E-FABP. After peritoneal injection of lipopolysaccharide, the immunoreactive cells in PALS progressively enlarged and became rounded in shape with a peak in size at 24 h postinjection and they eventually resumed the dendritic form at 48 h postinjection. Within each of the enlarged immunoreactive cell perikarya were included small immunonegative apoptotic cells, presumptive lymphocytes. Taken together, E-FABP is useful as a marker for dendritic cells in the splenic white pulp, and may be involved through combination with fatty acids in antigen presentation and retention as well as in cytokine production.

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Keywords Fatty acid binding protein · Dendritic cell · Spleen · Mouse · Epidermal

Introduction

Fatty acid binding proteins (FABPs) are cytosolic non-enzymatic proteins of low molecular weight, about 15 kDa, which are capable of binding long chain fatty acids. Several possible roles have been assigned to these FABPs: enhancement of the cellular uptake of fatty acids and their transport, compartmentation of intracellular fatty acid storage, modulation of the activity of enzymes involved in fatty acid metabolism, and protection of cellular enzymes and membranes from detrimental effects of fatty acids (Glatz et al. 1993; Coe and Bernlohr 1998). Multiple isoforms of FABPs have been identified and named by their initial sites of detection: liver (L), intestinal (I), heart (H), brain (B), skin or epidermis (S or E), and adipocyte (A) (Gordon et al. 1983; Alpers et al. 1984; Hunt et al. 1986; Siegenthaler et al. 1993; Feng et al. 1994; Kurtz et al. 1994). However, subsequent studies have demonstrated that individual FABP species show much wider tissue distribution than their original isolation sites (Iseki et al. 1988; Veerkamp et al. 1990; Watanabe et al. 1991; Owada et al. 1996a, b, 1997, 2001, 2002a).

Among various FABPs, epidermal-type FABP (E-FABP) has recently been localized in alveolar macrophages and thymic epithelial cells by us (Owada et al. 2001, 2002a). Because the two cell species have roles in the immune reaction, it is reasonable to speculate a possible localization of E-FABP in some cell species of immune organs such as the spleen. This led us to examine the detailed localization of E-FABP in the spleen of mice in the present study. As a result, E-FABP was specifically localized in the dendritic cells and follicular dendritic cells of the white pulp, but not of the marginal zone or the red pulp, and the phagocytosis of apoptotic cells, presumptive lymphocytes, by dendritic cells after lipopolysaccharide (LPS) administration was clearly demonstrated for the first time in vivo by immunoreactivity for

E-FABP as a specific marker for the cells at the levels of immunolight and immunoelectron microscopy.

Materials and methods

Male C57BL/6 mice at postnatal week 10 were used in this study. The generation of E-FABP knockout mice is described elsewhere (Owada et al. 2002b). They were maintained under normal laboratory conditions.

For Western blotting, the cytosolic fraction was prepared by homogenization of the spleen in 10 vol ice-cold buffer containing 250 mM sucrose, 25 mM KCl, 50 mM TRIS-HCl (pH 7.5), and 4 mM $MgCl_2$. Supernatants of the homogenates after centrifugation at 10,000 *g* for 10 min were analyzed by 11% SDS-PAGE under reducing conditions. Proteins in the gel were electroblotted onto a nitrocellulose membrane. The membrane was incubated with rabbit polyclonal anti-E-FABP antibody at a concentration of 0.5 μg/ml in phosphate-buffered saline (pH 7.4) containing 0.1% Tween 20 (TPBS).

For immunohistochemistry, individual mice were perfused under Nembutal anesthesia through the heart with 4% buffered paraformaldehyde. The spleens were extirpated and immersed overnight in a phosphate buffer containing 30% sucrose. Sections, 20 μm in thickness, were cut on a cryostat and incubated with individual antibodies at appropriate concentrations for 12 h at 4°C. Antibodies employed in this study include the E-FABP antiserum at a concentration of 0.5 μg/ml, and a monoclonal rat antibody against NLDC-145 (a kind gift from Dr. K. Matsuno, Dokkyo University, Japan) at 1:10 dilution. The specificity for the E-FABP antiserum was confirmed by negative staining in the spleen of E-FABP gene knockout mice. After incubation with the primary antibody, the sections were incubated with biotinylated anti-rabbit secondary antibody for single immunostaining or with a combination of anti-rabbit IgG–Alexa488 and anti-rat IgG–Alexa594 (Molecular Probes, USA) for double immunostaining. The sections for single immunostaining were subsequently visualized using the ABC (avidin–biotinylated peroxidase complex) system (Vector Laboratories, USA) with DAB as a substrate.

For immunoelectron microscopy, some of the sections were postfixed with 1% OsO_4 in 0.1% cacodylate buffer, pH 7.4, for 20 min after completion of the ABC procedure. They were embedded in Epon according to the conventional procedure and ultrathin sections were examined after brief staining with uranyl acetate.

For administration of LPS to create a model of endotoxemia, male C57BL/6 mice at postnatal week 10 were injected intraperitoneally with LPS (*E coli* serotype; Wako, Japan; 5 μg/g body weight) in saline. Three mice were killed under ether anesthesia at 0, 1, 3, 6, 24, and 48 h postinjection, and spleens were rapidly removed and immersed in 4% paraformaldehyde fixative for immunohistochemistry.

For TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridyl triphosphate nick-end labeling) combined with E-FABP immunohistochemistry, cryosections of spleen 24 h after LPS injection were prepared. The sections were incubated in a solution containing 100 mM sodium cacodylate (pH 7.0), 1 mM $CoCl_2$, 50 μg/ml gelatin, 10 nM/ml biotin-16-dUTP (Roche, Germany), and 100 U terminal deoxynucleotidyl transferase (Takara, Japan). After several washes with TPBS, sections were incubated with E-FABP antiserum followed by incubation with anti-rabbit IgG–Alexa488 (Molecular Probes) and anti-biotin IgG–Alexa594 (Molecular Probes). The sections were mounted with Gel/Mount (CosmoBio, Japan) and observed by confocal laser scanning microscope (Leica, Germany).

Results

A single band at 15 kDa in Western blotting which corresponded to the original E-FABP protein was detected in the mouse spleen, and no immunoreactive bands were detected in the spleens from the E-FABP gene knockout mice as expected (data not shown).

Histology of in situ spleen from adult mice without any external stimuli

In immunolight microscopy of the spleen of adult mice, intense E-FABP immunoreactivity was found in numerous cells and their tapering and radiating processes, and they appeared singly in the periarterial lymphoid sheaths (PALS) representing the T-cell areas (Fig. 1A, B) and also in the follicles representing the B-cell areas of the white pulp (Fig. 1A, C). This specific immunostaining revealed a difference in morphology between the immunoreactive cells in PALS and those in the follicles: the former had more voluminous perikarya with stumpy and short processes which were less frequently contiguous with those of adjacent immunoreactive cells (Fig. 1B), while the latter had smaller perikarya with longer and delicate processes which formed immunoreactive networks by contiguity with adjacent immunoreactive cell processes (Fig. 1C).

As a next step, we attempted to examine whether or not E-FABP-immunoreactive cells are also immunoreactive for NLDC-145, a membranous antigen specific to CD8(+) dendritic cells. In this regard it should be noted that the immunoreactivity for NLDC-145 is only obtained sufficiently in specimens that have been preserved by acetone fixation, and it is markedly attenuated in specimens preserved by aldehyde-based fixatives, while E-FABP immunoreactivity is hardly detected in specimens fixed with acetone. Based on this methodological limitation, double immunostaining for these two antibodies in spleens preserved with the regular paraformaldehyde fixation was performed in one and the same section, and cells double immunostained with the two antibodies were certainly found in PALS (Fig. 1D–F). In addition, when sections from spleens fixed with paraformaldehyde and those fixed with acetone were immunostained with antibodies for E-FABP and NLDC-145, respectively, the immunoreactive patterns for the two different antibodies were similar to each other in terms of their cell shape and population density in PALS (data not shown). On the other hand, no immunoreactive cells of such dendritic forms as described above were found in the marginal zones or the red pulp, although endothelial cells of almost all capillaries in the red pulp were moderately to intensely immunoreactive for E-FABP (Fig. 1A). None of cells with round profiles representing lymphocytes were immunoreactive for E-FABP.

In immunoelectron microscopy, the immunoreactive cells in both PALS (Fig. 2A, B) and the follicle (Fig. 2C) of the splenic white pulp from mice without LPS

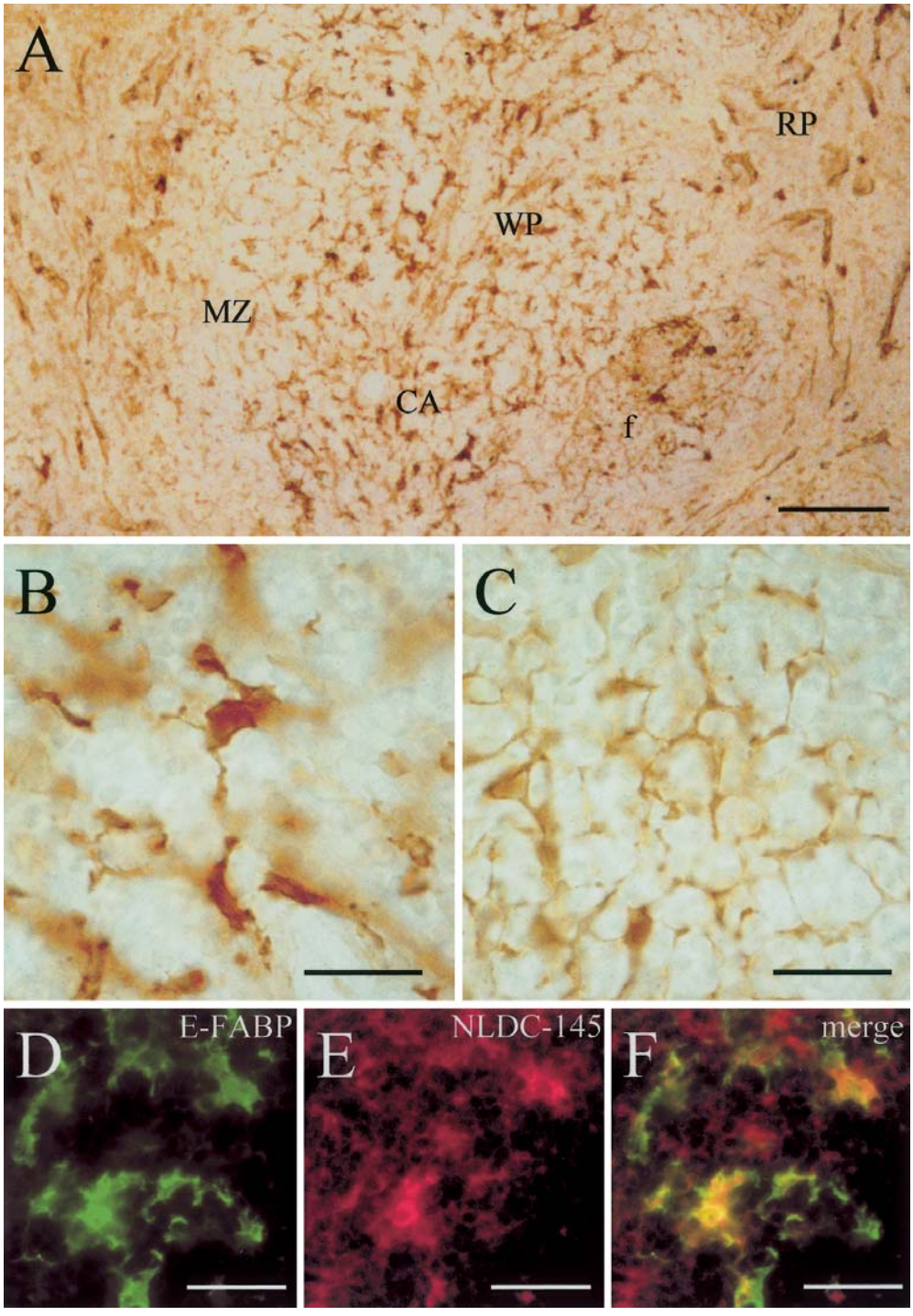

Fig. 1A–F Light micrographs of epidermal-type fatty acid binding protein (E-FABP)-immunoreactive cells in the mouse spleen at postnatal week 9 (**A**). High power magnification micrographs of E-FABP-positive cells in the periarterial lymphoid sheath (PALS; **B**) and follicle (**C**) of the splenic white pulp. **F** Superimposed image of the micrographs **D** (E-FABP) and **E** (NLDC-145) showing that E-FABP-positive cells are co-immunostained for NLDC-145, a marker of dendritic cells in the PALS. Because of a marked attenuation of NLDC-145 immunoreactivity in the aldehyde-fixed spleen, its immunoreactivity may not be discerned clearly in some processes of the cells strongly immunoreactive for E-FABP. *CA* Central artery, *f* follicle, *MZ* marginal zone, *RP* red pulp, *WP* white pulp. *Bars* 100 μm in **A**; 20 μm in **B**, **C**; 50 μm in **D–F**

treatment had a nucleus of oval shape with relatively few heterochromatins and contained regular organelles including mitochondria, Golgi apparatus, and rough endoplasmic reticulum. Lysosomes were relatively few and small in general although a few medium- to large-sized lysosomes might be seen in some of the immunoreactive cells. Immunopositive cell processes partially enclosed adjacent immunonegative lymphocytes whose nuclei were rich in heterochromatins. The immunoreactive material was localized densely in the cytoplasm and lightly in the nucleus, but not within any membranous cell organelles. Some of the processes were short while some others were extended for a rather long distance among lymphocytes. Such long processes were much more commonly found and thinly extended for immunopositive cells in the follicle than PALS (Fig. 2C). On the other hand, immunonegative cells having an oblong nucleus and slender processes were often found adjacent to the

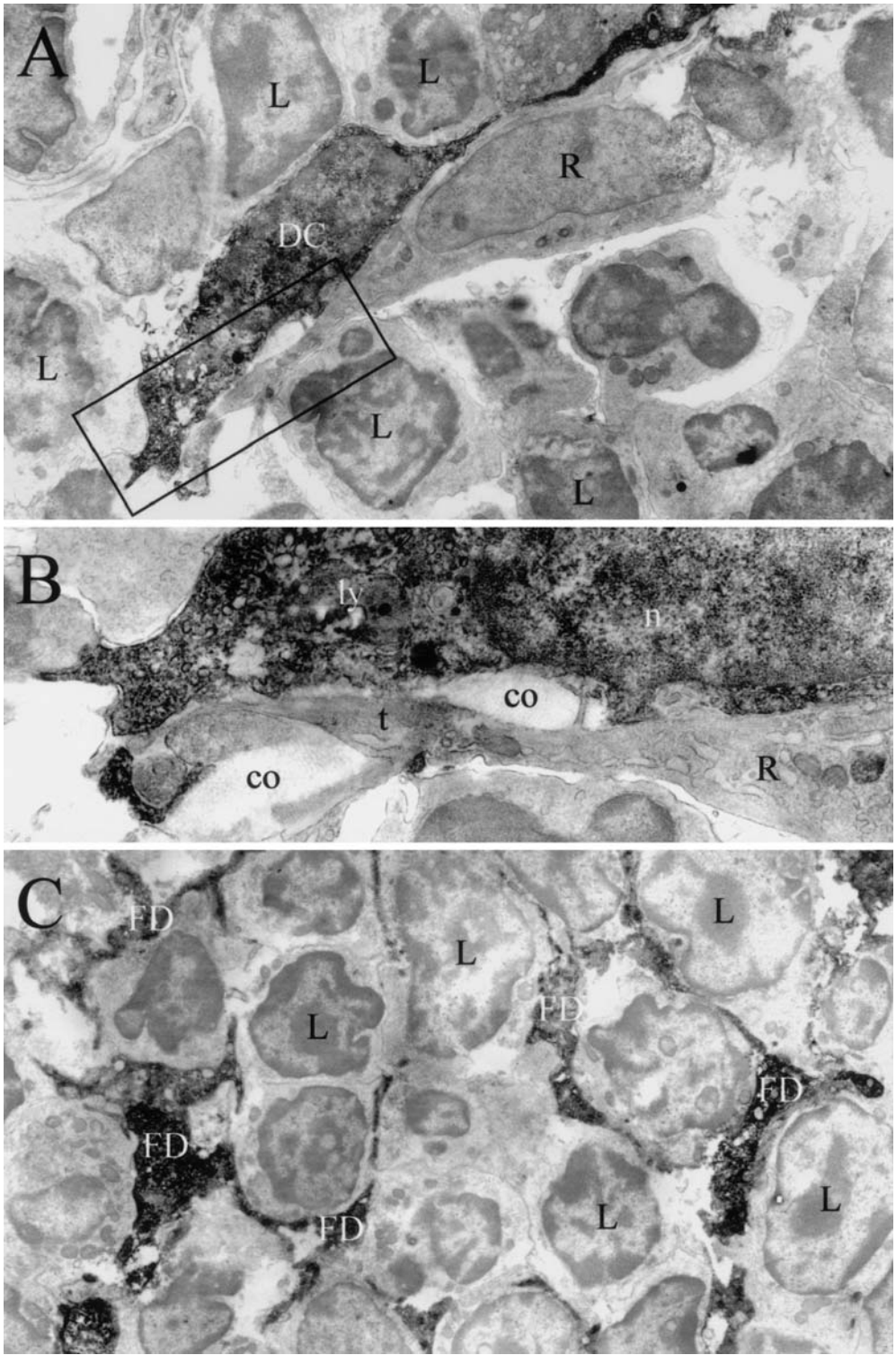

Fig. 2A–C Electron micrographs of E-FABP-immunoreactive cells in PALS (**A**, **B**) and follicle (**C**) of the spleen. An enlarged image of the area enclosed by a *rectangle* in **A** is shown in **B**. Note the immunoreactive dendritic cells (*DC*) with processes which enclosed immunonegative lymphocytes (*L*) and also note the immunonegative reticular cell having tonofilaments (*t*) within the cytoplasm. *co* Collagenous fibers, *DC* dendritic cell, *FD* follicular dendritic cell, *ly* lysosome, *n* nucleus, *R* reticular cell. Magnification approximately ×5,500 for **A**, ×12,000 for **B**, and ×4,500 for **C**

immunoreactive cells, and they were characterized by well-developed bundles of intermediate filaments, a relative paucity of cell organelles, and direct association with bundles of extracellular collagenous fibrils (*R* in Fig. 2A, B).

When E-FABP antibody was applied to sections of the spleen from E-FABP gene knockout mice whose phenotype in the skin had already been reported by us (Owada et al. 2002b), no immunoreactive cells were discerned in any portions of the sections (data not shown), indicating that the immunoreactivity in the spleen represents the authentic E-FABP.

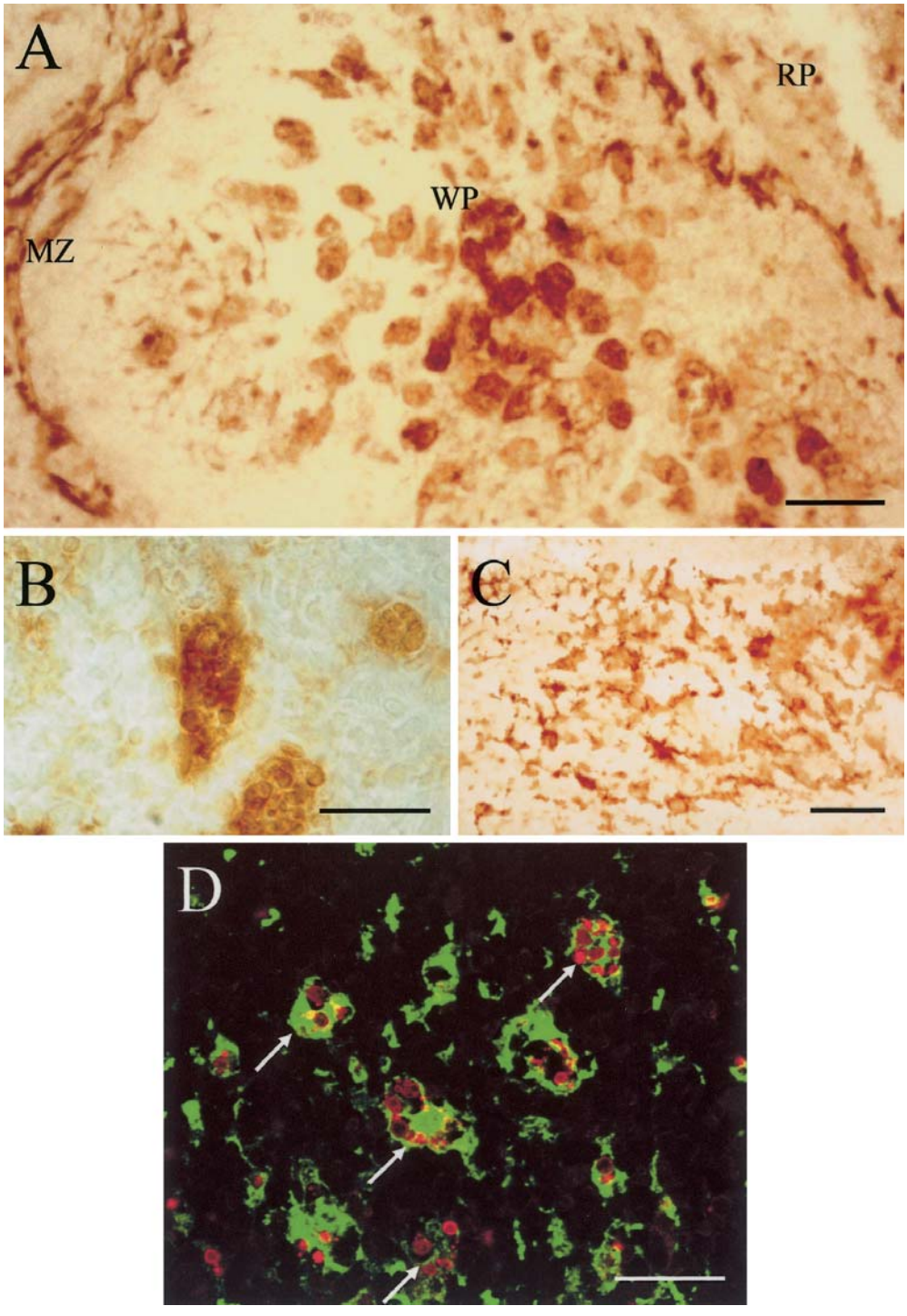

Fig. 3A–D Light micrographs of E-FABP-immunoreactive cells in the mouse spleen after lipopolysaccharide (LPS) administration. Note that E-FABP-positive cells were enlarged and rounded in shape 24 h after LPS administration (**A**, **B**) and they eventually recovered their appearance of the preinjection state at 48 h postinjection (**C**). E-FABP-positive cells (*green color*) engulfing the TUNEL-positive cells (*red color*) are shown by *arrows* in **D**. *MZ* Marginal zone, *RP* red pulp, *WP* white pulp. *Bars* 100 μm in **A**, **C**; 20 μm in **B**; 40 μm in **D**

Histological responses to LPS of in situ spleen

In immunolight microscopy, the immunoreactive cells in PALS were progressively enlarged and rounded in shape, and their size reached a peak ranging from 30 to 50 μm 24 h postinjection (Fig. 3A, B), although no marked changes in the cell population density of the immunoreactive cells were discerned throughout the time course. Thereafter the immunoreactive cells tended to take again the dendritic form and eventually the appearance of the preinjection state was recovered at 48 h postinjection (Fig. 3C). Several tiny immunonegative structures of round shape appeared to be included within each of the enlarged immunoreactive cell perikarya, resulting in a honeycombed appearance of the cells (Fig. 3B). When costained for TUNEL reaction and E-FABP immunoreactivity, all the enlarged cells immunoreactive for E-FABP were simultaneously positive for the TUNEL reaction

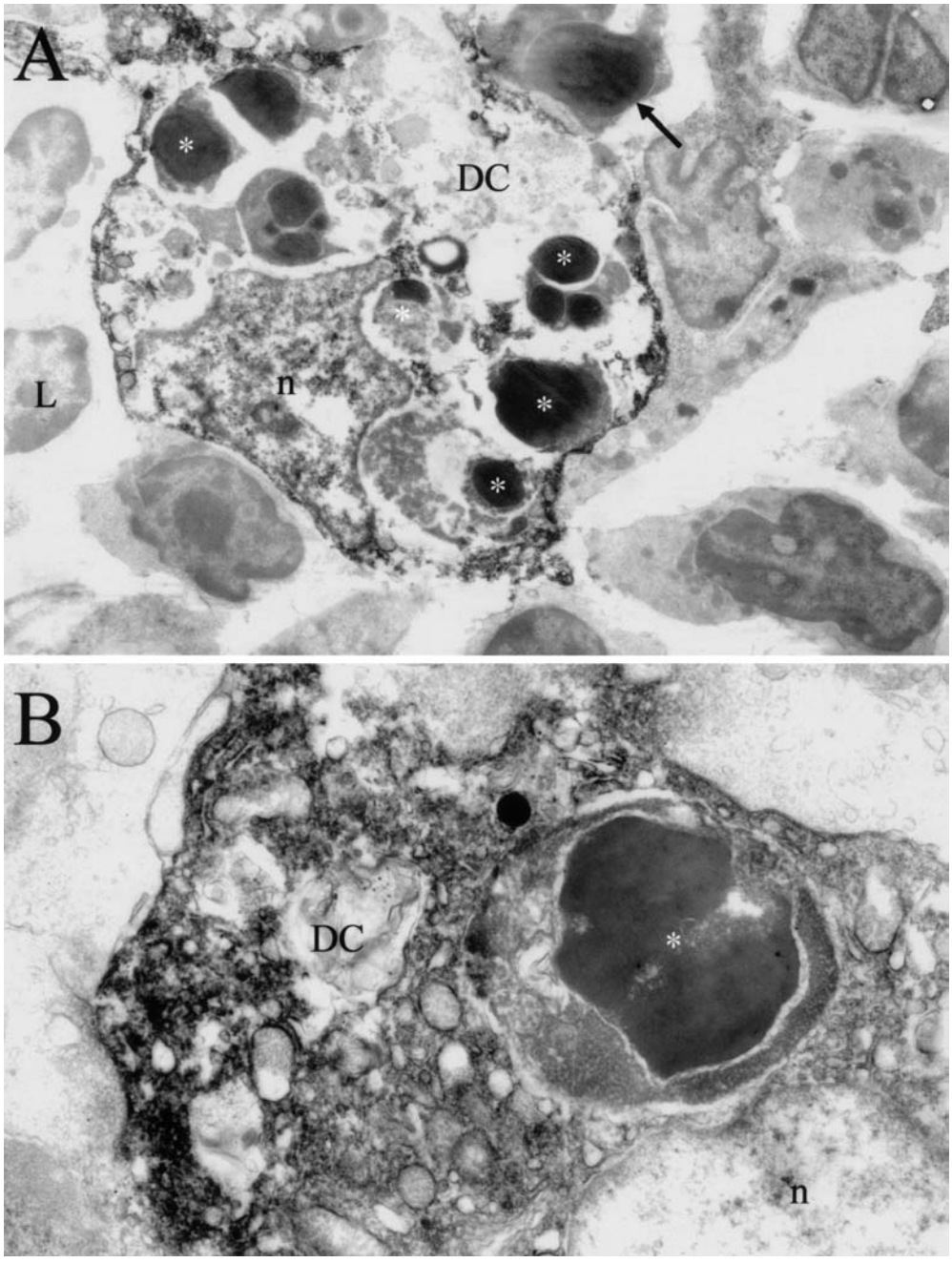

Fig. 4A, B Electron micrographs of E-FABP-immunoreactive cells in PALS (**A**, **B**) at 24 h postinjection of LPS. Note that E-FABP-positive cell includes phagocytosed apoptotic cells with chromatin-condensed nuclei (*asterisks*) within the cell bodies. Also note that apoptotic cells (*arrows* in **A**) are sometimes found in direct apposition to the enlarged immunoreactive cells. *DC* Phagocytosing dendritic cell, *L* lymphocyte, *n* nucleus. Magnification approximately ×5,500 for **A** and ×18,000 for **B**

(Fig. 3D). On the other hand, most of the immunoreactive cells in the follicles maintained a slender morphology with extensive cytoplasmic extension (Fig. 3A).

In immunoelectron microscopy, the immunoreactive cells of mice treated with LPS had an irregular-shaped nucleus whose matrix exhibited the immunoreactivity, and they enclosed multiple oval or oblong structures with their cytoplasmic processes or even included them within the cell bodies (Fig. 4A, B). The enclosed structures, with a size similar to or smaller than adjacent lymphocytes, were delineated with the unit membranes and were composed of two elements: one exhibited a rather homogenously high electron density (*asterisks* in Fig. 4A) while the other exhibits a much lower density and a profile of association with or surrounding the former, and mitochondria and membranous structures might be discerned in some of the latter (*asterisk* in Fig. 4B). Immunonegative lymphocytes having a condensed nucleus were sometimes found in direct apposition to the enlarged immunoreactive cells (*arrow* in Fig. 4A).

Discussion

E-FABP as a marker for dendritic cells in the white pulp

Judging from their dendritic shape and enclosing relation to adjacent lymphocytes together with almost the same patterns of cell distribution as those of NLDC-145-immunoreactive cells, E-FABP-immunoreactive cells in the splenic PALS are identified as dendritic cells that are CD8a(+) (Leenen et al. 1998). This identification is further supported by the simultaneous detection of E-FABP-immunonegative cells having a slender shape and intracellular tonofilaments, whose identification as resident reticular cells is highly likely (Weirsbowsky et al. 1982; McNagny et al. 1991; Farr et al. 1992; Dullmann et al. 2000). The rather thinly reticular pattern of the reticular cells demonstrated by those previous authors using several markers specific to reticular cells can be pointed out as a difference from the present immunoreactive pattern composed of rather voluminous somata and processes of the individual cells. On the other hand the immunopositive cells in the splenic follicles are identified as follicular dendritic cells based on their cytoplasmic ultrastructure and intimate association with adjacent lymphocytes by long and thinly delicate cytoplasmic extensions (Liu et al. 1996).

Since most, if not all, antigens known so far specific to the dendritic cells are membranous proteins, but not soluble ones, unlike E-FABP, and therefore their antigenicity is well preserved for immunohistochemistry only by fixatives containing acetone, the resulting immunoreactive cell images in tissue sections are insufficient to delineate the detailed contour of the cells clearly in light microscopy and to appreciate their ultrastructure. In contrast, E-FABP immunoreactivity was revealed in this study to be advantageous in demonstration of the detailed shape and cytological features of dendritic cells at light and electron microscope levels and this clear visualization made it possible to clarify the difference in the cell shape between the dendritic cells in PALS and the follicular dendritic cells in light and subsequent electron microscopy, although the slender features of the latter cell process had been individually reported (Szakal et al. 1985).

It is generally considered that the dendritic cells in PALS and the follicular dendritic cells in the follicle are discrete in origin, though it is still a much debated issue, the former being of hematopoietic origin and the latter of mesenchymal origin (Bell et al. 1999). This indicates that the expression of E-FABP is shared in common by the two dendritic cells. However, this is not the first case, and the same is true for CD40 and ICAM-1/CD54 which have been shown to be expressed in both dendritic cells and follicular dendritic cells as well as lymphocytes and fibroblasts (Liu et al. 1996). Whether this shared expression by the two cells of discrete origin represents a common role of E-FABP or its discrete roles in the two different cells remain to be elucidated. Regarding the functional significance of E-FABP, the absence of E-FABP immunoreactivity in CD8(−) dendritic cells at the border of splenic marginal zone and the red pulp should be also pointed out. This latter cell type represents a much larger population than CD8(+) dendritic cells in PALS (Agger et al. 1992; Leenen et al. 1998). The absence of E-FABP immunoreactivity in the CD8(−) dendritic cells suggests that E-FABP-immunoreactive CD8(+) dendritic cells, different from the CD8(−) cells, play some specific roles in terms of fatty acid-related metabolism and signal transduction, or that the CD8(−) cells have a potential of expressing E-FABP, but inhibit it under normal in vivo conditions. For examination of the latter possibility, the isolation in vitro of the two cells and stimulation of them with some appropriate extrinsic stimuli remain to be elucidated, although in situ stimulation by LPS failed in detection of such a potential in the present study.

Since the present study disclosed no immunoreactivity for B-, H-, or A-FABP in any splenic dendritic cells (data not shown), and because E-FABP among other FABPs shows preference in binding with saturated fatty acids to unsaturated ones (Hanhoff et al. 2002), although it binds almost all long chain fatty acids, possible roles specific to the CD8(+) dendritic cells and follicular dendritic cells may be related to their high requirement for saturated fatty acids, rather than that for fatty acids in general.

Active phagocytosis of LPS-induced dying cells by dendritic cells in PALS in situ

Judging from positive TUNEL reaction products on the enlarged immunoreactive cells in response to LPS administration in light microscopy, and ultrastructural features of apoptotic nuclei and cytoplasm within the enlarged immunoreactive cells, together with the finding that the enclosed structures were of a size similar to or smaller than adjacent lymphocytes, it is highly likely that the apoptotic cells are adjacent lymphocytes responding to LPS, and not dendritic cells as suggested by previous authors (De Smedt et al. 1996). While previous studies have presented evidence that splenic dendritic cells in vitro phagocytose dying cells (Rubartelli et al. 1997; Albert et al. 1998; Huang et al. 2000; Sauter et al. 2000; Steinman et al. 2000), it has recently been shown that only CD8(+) dendritic cells in situ take up syngeneic lymphocytes and tumor cell lines after induction of apoptosis ex vivo by exposure to osmotic shock and subsequent injection back into the mice (Iyoda et al. 2002). In compatibility with their finding, the present study represents the first demonstration at both light and electron microscope levels of the dendritic cells in situ in the splenic white pulp which phagocytose adjacent apoptotic lymphocytes in response to LPS. This first demonstration is made possible because, as stated above, E-FABP immunoreactivity is a histological marker for dendritic cells in PALS much better than any other counterparts in terms of cytological preservation and because the immunoreactivity in the cells does not faded away markedly after LPS administration. The absence of any marked change in the number of E-FABP-immuno-

reactive cells in the white matter throughout the time course of the LPS administration is another basis for the present interpretation that the dendritic cells originally immunoreactive for E-FABP remain in the white pulp, maintain their immunoreactivity, and exhibit active phagocytosis in response to LPS. For the same reason, it is unlikely that some dendritic cells in the marginal zone move into the PALS and join the population of E-FABP-immunoreactive dendritic cells which exhibit the phagocytotic activity in response to LPS administration, although there has been an interpretation that LPS can cause dendritic cells in the marginal zone to migrate into the white pulp and further out of the domain based on the finding that the number of splenic dendritic cells immunoreactive for such popular markers for the cell species as CD11c and NLDC-145 progressively decrease after LPS administration (De Smedt et al. 1996).

In order to further understand the functional significance of E-FABP in the dendritic cells and the follicular dendritic cells in the white pulp, the analyses of spleens from E-FABP gene knockout mice recently generated by us (Owada et al. 2002b) is crucial and which is underway in our laboratory.

Acknowledgements This work was supported by grants from the Ministry of Education, Science, and Culture of Japan, numbers 14370002 and 14657623 (to H.K.) and number 14580720 (to Y.O.).

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