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Abnormal development and differentiation of macrophages and dendritic cells in viable motheaten mutant mice deficient in haematopoietic cell phosphatase

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Summary. In mice homozygous for the 'viable motheaten' (*me^v*) mutation, numbers of macrophage progenitor cells, particularly monocytes, were markedly increased in the bone marrow and spleen. Increased mobilization of these precursor cells to peripheral tissues and their differentiation to macrophages were evidenced by striking increases in macrophage numbers. Immunohistochemical double staining of tissue sections and flow cytometry analyses of single cell suspensions from these mice demonstrated CD5 (Ly-1)-positive macrophages in the peritoneal cavity, spleen and other tissues. Ly-1-positive macrophage precursor cells were demonstrated in the peritoneal cavity of the *me^v* mice and developed in the omental milky spots. The development of marginal metallophilic and marginal zone macrophages was poor in the splenic white pulp and related macrophage populations were absent in the other lymphoid tissues. The numbers of epidermal Langerhans cells in the skin and T cell-associated dendritic cells in the thymic medulla, lymph nodes, and the other peripheral lymphoid tissues were decreased. However, increased numbers of dendritic cells accumulated in the lungs, liver, and kidneys. These abnormalities in development and differentiation of macrophages and dendritic cells may be ascribed to the deficiency in haematopoietic cell SHP-1 tyrosine phosphatase or may be a secondary consequence of abnormal microenvironments, (either constitutive or in response to inflammatory stimuli) in the haematopoietic and lymphopoietic organs and tissues of these mice.

Keywords: immunohistochemistry, flow cytometry, mouse, immunodeficiency, monocyte/macrophages, dendritic cells

Introduction

Mice homozygous for the recessive allelic mutations motheaten (*me*) and viable motheaten (*me^v*) on

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Chromosome 6 have mutations in the gene encoding an SHP-1 tyrosine phosphatase termed haematopoietic cell phosphatase. The official gene symbols for the *me* and *me^v* mutations are *Hcph^{me}* and *Hcph^{me-v}*, respectively. (We will use the abbreviated gene symbols, *me* and *me^v* throughout). These mutations cause severe haematopoietic dysregulation, a severe combined immunodeficiency and systemic autoimmune disease (Shultz *et al*. 1993). Homozygotes (*me* and *me^v* mice) can be identified by the development of skin lesions by 2 or 4–5 days after birth, respectively, giving the animals the characteristic 'motheaten' appearance. They die of pneumonia at a mean age of 3 and 9 weeks, respectively (Green & Shultz 1975; Shultz *et al*. 1984). The mutant mice exhibit multiple haematopoietic abnormalities including impaired T and B lymphopoiesis, myelomonocytopoiesis and macrophage accumulation (Green & Shultz 1975; Shultz & Green 1976; Shultz 1987; van Zant & Shultz 1989; Shultz *et al*. 1984, 1993).

The mutant mice reveal prominent haematopathological abnormalities including a dramatic increase of granulocyte/macrophage colony-forming cells (GM-CFC) and erythroid CFC (E-CFC) in the spleen, decreases of these precursor cells in the bone marrow and progressive increases of monocyte/macrophages and neutrophilic leukocytes in various tissues, particularly in the skin and lungs (Green & Shultz 1975; van Zant & Shultz 1989; Mlinaric-Rascan *et al*. 1994; McCoy *et al*. 1982, 1983; Shultz *et al*. 1984, 1993). These abnormalities are associated with impairments of microenvironment in the bone marrow of *me^v* mice (van Zant & Shultz 1989). In addition, accelerated rate and unusual growth characteristics of the monocyte/macrophage production in the spleen of *me* mice were demonstrated *in vitro* (McCoy *et al*. 1982, 1983). In previous studies, myelomonocytic proliferation and macrophage accumulation were reported in various tissues of *me^v* mice (Green & Shultz 1975; Shultz *et al*. 1984; van Zant & Shultz 1989; Mlinaric-Rascan *et al*. 1994; McCoy *et al*. 1982, 1983). More recently, CD5 (Ly-1)-positive macrophages were detected in the spleen of *me^v* mice (Borrello & Phipps 1996). However, changes in number of monocyte-derived macrophages, tissue macrophages, and Ly-1-positive macrophages have not been examined in detail in various tissues of *me^v* mice. In addition, little is known about the distribution and localization of macrophages and their subpopulations in various tissues of *me^v* mice. Although previous studies demonstrated a marked reduction of epidermal Langerhans cells in *me* and *me^v* mice (Sprecher *et al*. 1990), the distribution, development, and differentiation of nonlymphoid dendritic cells in the thymic medulla and peripheral lymphoid tissues of the mutant mice remain unknown.

Recently, many hybridoma cell lines producing anti-mouse monoclonal antibodies specific for monocyte/macrophages, tissue-fixed macrophages, macrophage subpopulations, macrophage precursors, and dendritic cells have been developed. Using these monoclonal antibodies, we examined the distribution and localization of macrophages and their related cells and identified their precursor cells by immunohistochemical or flow cytometric analysis, utilizing a panel of monoclonal antibodies for macrophages, their subpopulations, macrophage precursor cells, and dendritic cells in *me^v* mice and normal littermates (Table 1). Using these monoclonal antibodies in various combinations, immunohistochemical or flow cytometric double staining protocols were performed to characterize immunophenotypic features of the cells in *me^v* mice and normal littermates.

Materials and methods

Animals

C57 BL/6 J strain mice heterozygous for the *me^v* mutation were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and fed at the Laboratory Animal centre of Kumamoto University School of Medicine. Homozygous *me^v/me^v* mice and littermates were propagated by $+/me^v$ brother/sister matings of the strain and the littermate controls consist of two-thirds $+/me^v$ and one third $+/+$; both genotypes are indistinguishable.

Tissue preparation

Animals were sacrificed and spleen, thymus, lymph nodes, liver, lungs, kidneys, bone marrow and other various tissues were examined from both the *me^v* mice and normal littermates at 4 days after birth (week 0) or at age one week to 9 weeks. At each week, at least three mice were examined. For histopathological examination, part of these tissues was fixed in 10% buffered neutral formalin and the other part was fixed in 2% periodate-lysine-paraformaldehyde (PLP) fixative at 4 °C for 4 h.

Histopathology

Formalin-fixed tissues were embedded in paraffin and paraffin sections were stained with haematoxylin and eosin (HE) for histological examination.

Monoclonal antibodies

Anti-mouse monoclonal antibodies used in the present

Table 1. Immunoreactivities and immunoreactive cells of monoclonal antibodies used for immunohistochemistry (IH) or flow cytometry (FC)

Specificity of monoclonal antibodies	Immunoreactive cells (methods)
Thy-1	T lymphocytes (IH, FC)
CD5 (Ly-1)	Mature T lymphocytes, a small subset of B lymphocytes (IH, FC)
B220	B lymphocytes and lytically active subsets of lymphokine-activated killer cells (IH, FC)
F4/80	Promonocytes, monocytes, free or tissue-fixed macrophages, Kupffer cells, histiocytes, synovial A cells, microglia, phagocytes on the peri- and endosteal bone surfaces, epidermal Langerhans cells (IH, FC)
BM 8	Tissue macrophages (IH, FC)
ER-MP12	Immature myeloid precursors (GM-CFC), T cells (IH, FC)
ER-MP 20	M-CFC, monoblasts, promonocytes, monocytes, and immature macrophages (IH, FC)
ER-MP 58	M-CSF-responsive myeloid precursors (GM-CFC) (IH)
MOMA-1	Metallophilic macrophages in spleen and their related macrophage populations in the thymic medulla, lymph nodes, and peripheral lymphoid tissues, synovial A cells, and macrophages in the uterus or ovary (IH)
ER-TR 9	Marginal zone macrophages in spleen and their related macrophage population in thymic medulla, lymph nodes, and other organs (IH)
NLDC-145	Epidermal Langerhans cells, interdigitating cells, dendritic cells, thymic epithelia (IH)
MIDC- 8	Epidermal Langerhans cells, interdigitating cells, dendritic cells, keratinocytes (IH)
CD4	Helper/inducer T cells, delayed type hypersensitivity T cells (IH, FC)
CD8	Thymocytes, suppressor/cytotoxic mature T cells (IH, FC)

Concentration of monoclonal antibodies used is described in the text.

study were Thy-1 (Cedarlane Laboratories, Hornby, Canada), CD4 (Pharmingen, San Diego, CA, USA), CD8α chain (Chemicon, Temecula, CA, USA), B220 (Pharmingen, San Diego, CA, USA), F4/80 - (Serotec, Oxford), BM8, MOMA-1, ER-TR9, ER-MP12, ER-MP20, ER-MP58, NLDC-145, and MIDC-8 (BMA Biomedicals, August, Switzerland).

For flow cytometry, we used fluorescein isothiocyanate-conjugated F4/80, ER-MP20 (BMA Biomedicals), and Ly-1 (Becton Dickinson, Mountain View, CA, USA), and biotin-conjugated B220, ER-MP12, and F4/80 (Serotec). IgG2a and IgG2b used as blocking antibodies were purchased from Pharmingen. Table 1 shows the antigen specificity and immunoreactivity of the monoclonal antibodies.

Immunohistochemistry

PLP-fixed tissue specimens were washed with phosphate buffer saline (PBS) containing 10%, 15%, and 20% sucrose at 4°C for 4 h, and embedded in OCT compound (Miles, Elkhart, IN, USA). After freezing in dry ice-acetone, the tissues were cut into 5 μm-thick sections by a cryostat (Bright, Huntington). After inhibition of endogenous peroxidase activity by the method of Isobe *et al*. (1977), the cryostat sections were immunostained by the indirect immunoperoxidase method using monoclonal antibodies mentioned above (Umeda *et al*. 1996). As secondary antibodies, antirat immunoglobulin-horseradish peroxidase-linked F(ab')$_2$ fragment (Amersham, Poole, UK) diluted 1:100 according to the manufacture's instructions was used for Thy-1, B220, F4/80, BM8, MOMA-1, ER-TR9, NLDC-145, MIDC-8, CD4, CD8 alpha, ER-MP12, ER-MP20, or ER-MP58. After visualization with 3,3'-diaminobenzidine (Sigma Chemical Corp., St. Luois, MO, USA), the sections were counterstained with haematoxylin and mounted with malinol. As negative controls, the same procedures were performed omitting the primary antibodies.

For immunohistochemical double staining, cryostat sections were stained with F4/80, B220, ER-MP20, or ER-MP58 as primary antibodies by the method as described above. After visualization with 3,3'-diaminobenzidine as a brown colour, the sections were washed with 0.1 mol/l glycine/HCL buffer for 30 min at 3 times to remove first and second antibodies. Secondly, the sections were stained with Ly-1 as a secondary antibody for 2h. After washing with phosphate-buffered saline, they were incubated with rabbit antirat immunoglobulin (Serotec) at room temperature for 1 h. Then, they were washed with Tris-buffered saline and visualized with a mixture of 0.2 mmol/l naphthol AS-MX phosphate, 1 mmol/l fast blue BB salt, and 1 mmol/l levamisole (Sigma) in 50 mmol/l Tris/HCl buffer (pH 9.0) as a blue colour. To confirm the reaction specificity of these antibodies, we performed the control staining

with the first antibodies in the first and/or second step omitted. To examine the immunophenotype of dendritic cells more clearly, double stainings with ER-MP20 and MIDC-8 or NLDC-145 were performed as above.

Immunofluorescence staining and flow cytometry

The expression of cell surface antigens for lymphocytes, myeloid precursors, or macrophages was analysed by flow cytometry as described previously (Umeda *et al.* 1996). Cells (10^6) were prepared from bone marrow, spleen, thymus, or peritoneal wash out by using a syringe with FACS solution (phosphate buffer $+1\%$ bovine serum albumin $+0.05\%$ sodium azide) and filtering through nylon mesh. After blocking with rat IgG2a and IgG2b, the cells were stained directly with 50 μl of biotinylated monoclonal antibodies for 20 min on ice, and washed with PBS containing 1% foetal bovine serum (FBS). Afterward, the cells were incubated with phycoerythrin-conjugated streptavidin (Serotec, Oxford) and fluorescein isothiocyanate-conjugated monoclonal antibodies for 20 min and analysed on a FACS scan (Becton Dickinson). In the flow cytometric assays, 10 μg/million cells were used for F4/80 and 1 μg/million cells for the other antibodies as described previously (Umeda *et al.* 1996).

Cell enumeration

Numbers of cells positive for each monoclonal antibody were counted per 1 mm^2 in sections of various organs and tissues stained with various monoclonal antibodies. In each tissue, 10 or more sites were randomly selected, excluding apparently inflammatory lesions, and the numbers of positive cells were counted by the blinded counting procedure. The cell numbers listed on Figures are representative of at least three mice examined at each week.

Statistics

Significance of data was evaluated by Student-*t*-test. $P<0.05$ and $P<0.01$ were considered statistically significant.

Results

Immunohistochemical and flow cytometric analysis of monocyte/macrophage populations in various organs and tissues of viable motheaten mice

Figure 1 shows the numbers of F4/80-positive macrophages in the spleen, thymic medulla, liver, and lungs of

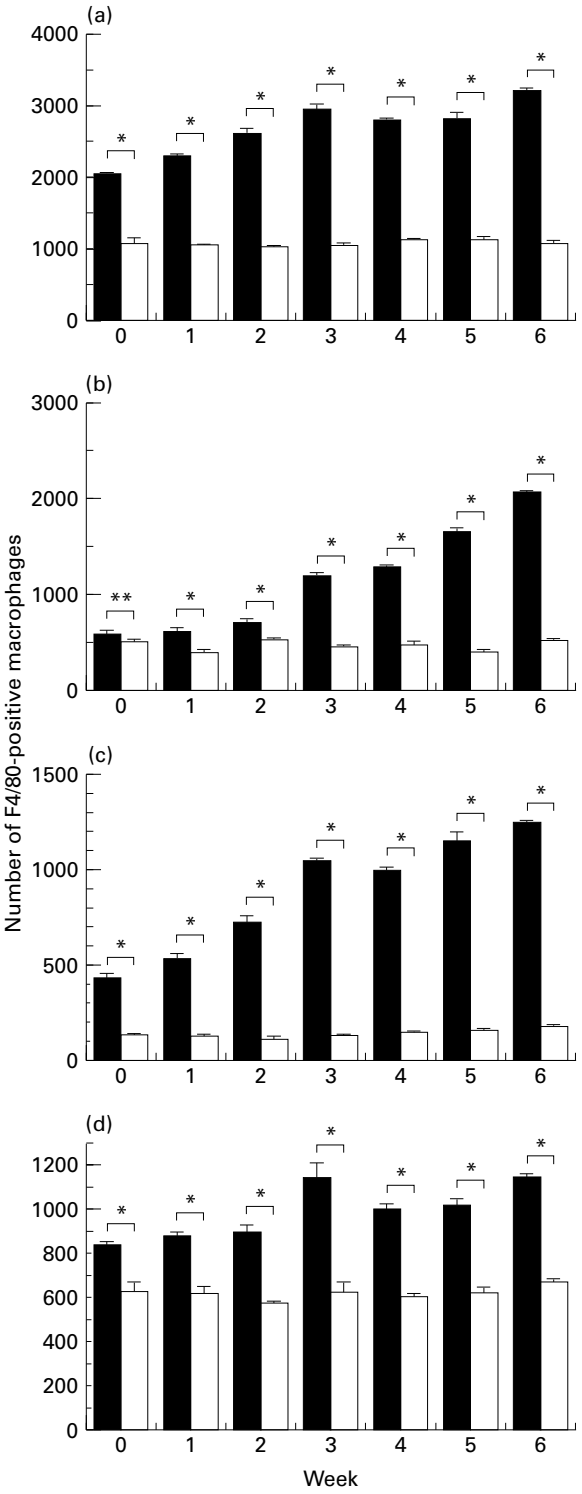


Figure 1. Numbers of F4/80-positive macrophages in (a) the spleen, (b) the thymus, (c) the lungs and (d) the liver of (■) *me*^{v} mice and (□) normal littermates at 4 days after birth (week 0) and at the age 1–6 weeks (*$P<0.01$,**$P<0.05$). Cell numbers ± SD are representative of at least 3 mice of each type examined at each age

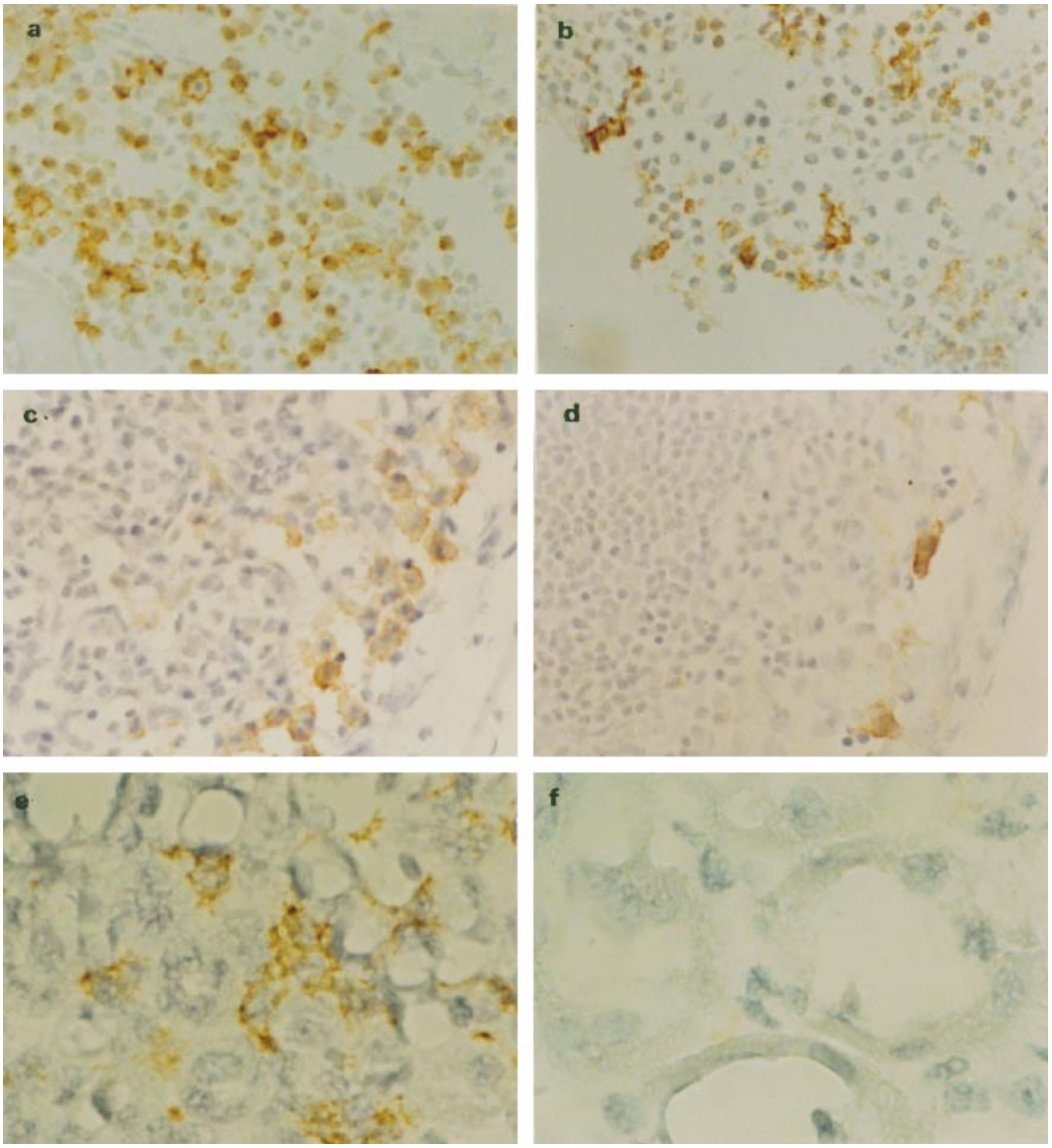
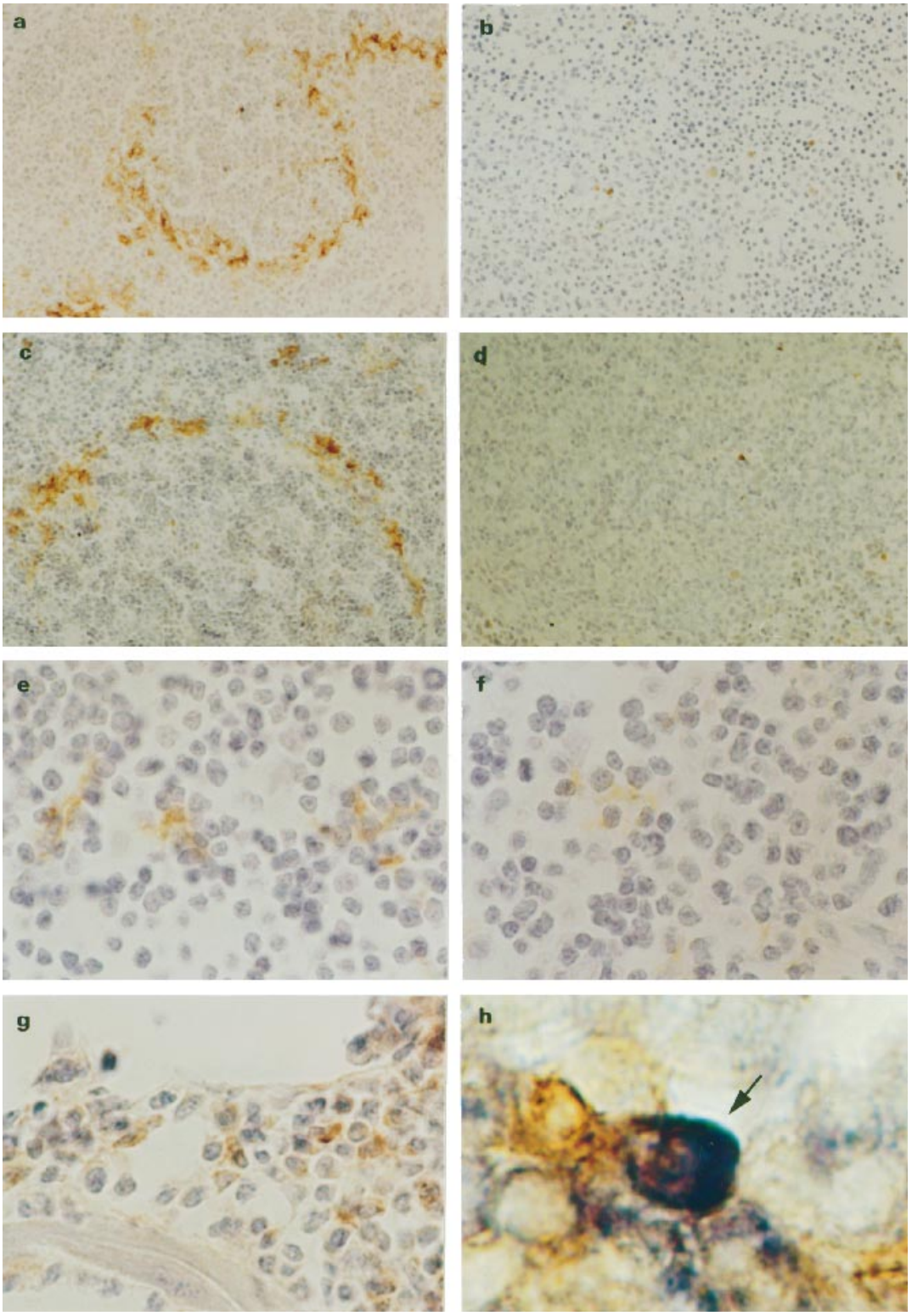


Figure 2. Distribution of F4/80- or BM8-positive macrophages in the bone marrow (a, b), lymph nodes (c, d) and renal interstitium (e, f) of *me*^{v} mice and normal littermates at the age of 3 weeks. Compared to the normal littermates (b, d, and f), numbers of macrophages are increased in the bone marrow (a), lymph nodes (c) and renal interstitium (e) in the *me*^{v} mice. Immunostaining with F4/80 (a-d) and MB8 (e-f). × 100 (a-d) and × 200 (e-f)

me^{v} mice and normal littermates from 4-5 days to 6 weeks of age. In the lungs of *me*^{v} mice, pneumonic lesions developed occasionally from 3 weeks after birth onward. The numbers of positive cells were counted in the pulmonary interstitium and alveolar walls free from the pneumonic lesions. In the mutamt mice, inflammatory cell infiltration also occurred in the spleen, lymph nodes, kidneys, joints, and skin from about 3 weeks after birth onward. In addition, extramedullary haematopoiesis in the liver of the *me*^{v} mice upto 3 weeks of age and it continued in the spleen thereafter. The lymph nodes were hypoplastic and showed inflammatory cell infiltration from 3 weeks after birth on. From this stage, the kidneys started to show mesangial proliferation and developed typical mesangioproliferative glomerulonephritis at several weeks of age, accompanied by inflammatory reaction by the interstitium.

Compared to the normal littermates, the numbers of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F4/80-positive macrophages were increased within a few days of age and became more numerously with age in all these tissues examined of the *me^v* mice. The increased numbers of F4/80-positive macrophages in these tissues of the *me^v* mice were more marked than those of BM8-positive macrophages (Fig. 2). In the *me^v* mice, F4/80- or BM8-positive macrophages were increased in several other organs and tissues, including bone marrow, lymph nodes, and renal interstitium (Fig. 2a, c & e), compared to normal littermate mice (Fig. 2b, d & f). In the *me^v* mice, the numbers of ER-MP20-positive cells and ER-MP58-positive cells in the bone marrow and spleen were also increased, compared to the normal littermates and these numerical increases were nearly similar to those of macrophages as shown in Fig. 1 (data not shown). In the liver and thymic medulla of normal littermates, there were low numbers of ER-MP20- or ER-MP58-positive monocytic precursor cells, whereas there were increased numbers of such cells in the *me^v* mice (data not shown). Numbers of ER-MP12-positive cells were also increased in these tissues of the *me^v* mice (data not shown).

In the mutant mice, the increased numbers of monocyte/macrophages and their precursors were confirmed in various tissues within a couple of days after birth and before the occurrence of inflammatory reactions. All these results suggest that macrophage precursor cells including monocytic cells are produced in the bone marrow and spleen, mobilized into the peripheral tissues in response to tissue environmental abnormalities or certain inflammatory stimuli, and differentiated into macrophages in loco.

Defective development and differentiation of certain macrophage populations in viable motheaten mice

Although the spleen was enlarged in the *me^v* mice, the white pulp showed atrophy, abnormal architecture, and ill-defined marginal zone, irregular distribution and decreased numbers of Thy-1- or B220-positive cells, and diffuse infiltration of Thy-1- or B220-positive lymphocytes into the red pulp. Compared to the normal littermates, numbers of CD8-positive cells in *me^v* mice were decreased. Those CD8-positive cells were distributed diffusely in the splenic red pulp of *me^v* mice. Many CD4-positive cells were also dispersed in the splenic red pulp of *me^v* mice, whereas they were mainly localized in the splenic white pulp of the normal littermates (data not shown).

In the normal littermates, MOMA-1-positive macrophages in the spleen first appeared in the marginal zone of the white pulp from one week of age onward and MOMA-1-positive cells developed abundantly in the whole circumference of the marginal zone by 3 weeks after birth (Fig. 3a). In normal littermates, ER-TR9-positive macrophages were localized in the outer marginal zone, these cells developed after birth, and their numbers reached the levels of adult mice at age 3 weeks after birth (Fig. 3c). Compared with the normal littermates, the development of MOMA-1- or ER-TR9-positive macrophages in *me^v* mice was poor. Numbers of MOMA-1-positive marginal metallophilic macrophages were extremely reduced in the marginal zone of splenic white pulp, and they were distributed sporadically in the splenic red pulp of *me^v* mice (Fig. 3b & d). In the normal littermates, in addition to their localization in the marginal zone of splenic white pulp, MOMA-1-positive macrophages developed in the thymic medulla, lymph nodes, synovial membrane, uterus, or ovaries (Fig. 3e). In the *me^v* mice, however, the development of MOMA-1-positive macrophages in all these tissues was poor and their numbers were markedly reduced (Fig. 3f). In the normal littermates, in addition to marginal zone macrophages in the spleen, ER-TR9-positive macrophages developed in the thymic medulla and lymph nodes. However, there were few ER-TR9-positive cells in these tissues of *me^v* mice. All these findings indicate defective development and differentiation of both MOMA-1- and ER-TR9-positive macrophage populations in various tissues of *me^v* mice.

Development and differentiation of Ly-1-positive macrophages in viable motheaten mice

Flow cytometric analysis revealed that 33.6% of the cells in the peritoneal cavity of *me^v* mice at 3 weeks of age were Ly-1- and F4/80-positive (Fig. 4). B220-and F4/80-double positive cells were 27.9% of the total peritoneal

Figure 3. Distribution and development of MOMA-1- or ER-TR9-positive cells in the marginal zone of white splenic pulp (a-d), thymic medulla (e, f) and lymph nodes (g, h) of *me^v* mice (b,d,f,h) and normal littermates (+/–)(a,c,e.g) at the age 3 weeks. In the normal littermates, MOMA-1-positive marginal metallophilic macrophages and ER-TR9-positive marginal zone macrophages are well developed in the spleen (a, c). neither macrophage populations are present in the spleen of *me^v* mice (b, d). in the thymic medulla, MOMA-1-positive macrophages are present in the normal littermates (e) and decreased in the mutant mice (f). NLDC-145-positive cells infiltrate in inflammatory cells of the pulmonary interstitium of the of *me^v* mice (g). h, An arrow indicates a doubly stained cell with NLDC-145 (blue) and ER-MP20 (brown). Immunostaining with MOMA-1 (a, b, e and f), ER-TR9 (c, d) and with NLDC-145 (g). h, double staining with NLDC-145 and ER-MP20. × 50 (a-d), × 200 (e-g) and ×500 (h)

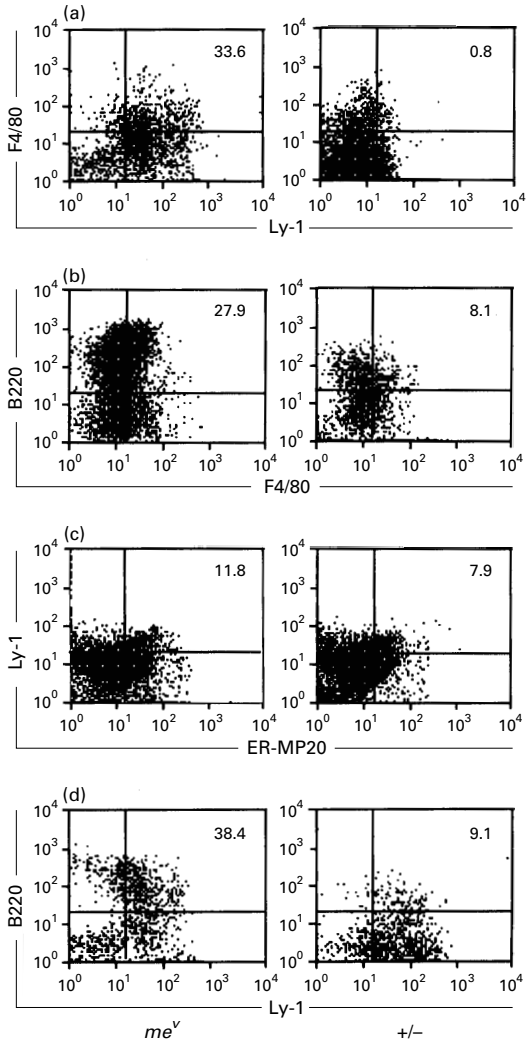


Figure 4. Flow cytometry analyses of peritoneal cell populations in *me*^{v} and ± normal littermate mice at 3 weeks of age. a, Two colour staining for Ly-1- and F4/80-positive macrophages. b, Two colour staining for B220- and F4/80-positive macrophages. c, Two colour staining for Ly-1- and ER-MP20-positive cells. d, Two colour staining for Ly-1- and B220-positive cells (B-1 cells)

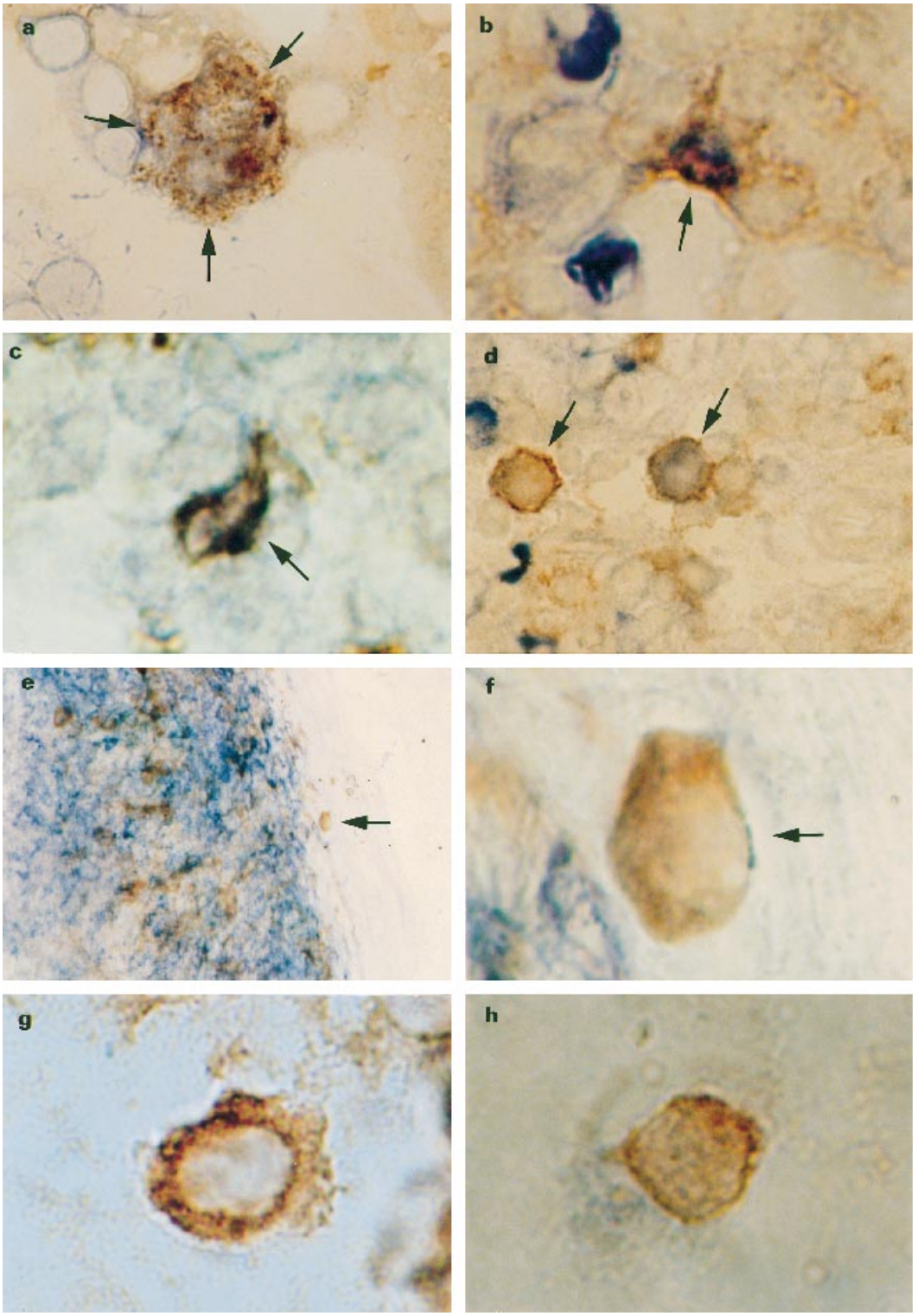
cells of the mutant mice (Fig. 4). In contrast, such Ly-1- and F4/80-double positive cells were not observed in normal littermate mice. Compared to the normal littermates, numbers of Ly-1- and B220-double positive cells (B-1 cells) were also increased in the peritoneal cavity of *me*^{v} mice (Fig. 4); 38.4% of the peritoneal cells were Ly-1- and B220-double positive. In normal littermates, double positive cells expressing F4/80 and Ly-1 or B220 were extremely rare in the peritoneal cavity. Flow cytometric analysis demonstrated that Ly-1- and F4/80-double positive macrophages and B220- and F4/80-double positive macrophages were 2.51% or 3.90% in the spleen of the *me*^{v} mice, respectively, and that Ly-1- and F4/80-double positive macrophages were 3.91% in the bone marrow of the mutant mice (data not shown).

Immunohistochemical double staining with Ly-1 and F4/80 or BM8 demonstrated the presence of Ly-1-positive macrophages in the peritoneal cavity, spleen, bone marrow, and lymph nodes of the *me*^{v} mice (Fig. 5a-d); however, no Ly-1-positive macrophages were detected in any tissues of the normal littermates. By light microscopy, no particular differences in size, cell density, and tissue structure of omental milky spots were found between the mutant mice and normal littermates. However, in the milky spots of the omentum, increased numbers of Ly-1-positive macrophages and their precursor cells doubly positive for Ly-1 and ER-MP20 or ER-MP58 were found in the *me*^{v} mice (Fig. 7e–h).

In *me*^{v} mice, Ly-1-positive macrophages were abundant in the peritoneal cavity and localized in the milky spots of omentum, where Ly-1-positive macrophages precursors were present. These data suggest that Ly-1-positive macrophages develop in the milky spots and migrate into the peritoneal cavity.

Development and distribution of dendritic cells in the epidermis and various lymphoid tissues of viable motheaten mice

Figure 6 shows changes in number of MIDC-8-positive dendritic cells in the splenic white pulp, thymic medulla, and lymph nodes of *me*^{v} mice and normal littermates from 4-5 days to 6 weeks after birth. In these tissues, although no significant difference in number of MIDC-8-positive dendritic cells between the *me*^{v} mice and normal littermates was found at 1 week after birth, numbers of MIDC-8-positive cells decreased gradually with age in the mutant mice. Similar reductions in number of NLDC-145-positive dendritic cells were found in the splenic white pulp and paracortical area of lymph nodes in the *me*^{v} mice. In agreement with the results of previous studies (Shultz 1987, 1988), the numbers of NLDC-145-positive epidermal Langerhans cells were decreased in the skin of *me*^{v} mice from the age 3 weeks onward, compared to the normal littermates (data not shown). In contrast, increased numbers of NLDC-145- or MIDC-8-positive dendritic cells infiltrated in the lungs, liver, and renal interstitium of *me*^{v} mice (Figs 3g & 6). In these tissues, infiltration of Thy-1-positive cells and macrophages was demonstrated in various degrees. In the kidneys of the mutant mice, the numbers of Thy-1-positive cells, B220-positive cells, and F4/80- or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BM8-positive macrophages were increased in the renal interstitium from 3 weeks after birth onward.

On immunohistochemical double staining with MIDC-8 (for dendritic cells) and ER-MP20 (for monocytic cells), dendritic cells were classified into two types; one is positive for MIDC-8 but not ER-MP20, and the other is double positive for both. In normal littermates and *me*^{v} mice, the former was found in lymphoid tissues, while the latter was occasionally detected in the lungs, liver, and renal interstitium of *me*^{v} mice, particularly in pulmonary interstitium (Fig. 5h). These data indicate that the dendritic cells appearing in the lungs, liver, and renal interstitium of *me*^{v} mutant mice share an immunophenotypic feature of monocytic cells in distinction to those in the skin and lymphoid tissues.

Discussion

The present investigation revealed marked infiltration and accumulation of monocyte/macrophages in various tissues of *me*^{v} mice. Moreover, abnormal macrophage populations in *me*^{v} mice included the enhanced production of macrophage precursor cells in the bone marrow and spleen, the presence of Ly-1-positive macrophages in the peritoneal cavity, spleen, bone marrow, and some other tissues, defective development of MOMA-1-positive marginal metallophilic and ER-TR9-positive marginal zone macrophage populations in the spleen, and absence of MOMA-1- or ER-TR9-positive macrophage populations in the other tissues. Furthermore, *me*^{v} mice showed reduced numbers of epidermal Langerhans cells, dendritic cells in the thymic medulla, lymph nodes, and peripheral lymphoid tissues, while infiltration of dendritic cells was found in the lungs, liver, and kidneys of the mutant mice.

In agreement with the data in previous studies (McCoy *et al*. 1982, 1983; Mlinaric-Rascan *et al*. 1994), the numbers of F4/80- or BM8-positive macrophages were increased in various tissues of *me*^{v} mice and the numbers of F4/80-positive monocyte/macrophages were increased to higher levels than that of BM8-positive tissue macrophages. Therefore, although tissue macrophages were increased in various tissues of the *me*^{v} mice within a few days after birth or before the occurrence of inflammatory lesions, the infiltration and numerical increment of monocyte/macrophages were most striking in most of the tissues from about 3 weeks of age onwards and became prominent in the inflammatory lesions. A recent study reported that macrophages in *me* and *me*^{v} mice showed increased proliferative responses to granulocyte/macrophage colony-stimulating factor (GM-CSF) but not macrophage colony-stimulating macrophage colony-stimulating factor (M-CSF), suggesting that GM-CSF is a major factor for macrophage development in the mutant mice (Jiao *et al*. 1997). In the current study using ER-MP20 and ER-MP58 antibodies, we demonstrated an increased production of ER-MP20-positive cells (monocytes, promonocytes, monoblasts, macrophage colony-forming cells (M-CFCs)) and ER-MP58-positive cells (M-CSF-responsive GM-CFCs) in bone marrow, spleen, liver and thymic medulla of *me*^{v} mice. The expansion of myelomonocytic population in the haematopoietic tissues of the mutant mice is associated with infiltration and accumulation of monocyte/macrophages in various tissues. These changes may result in an enhanced sensitivity of monocyte/macrophages to GM-CSF. Dysregulation of M-CSF receptor-mediated signal transduction may then induce enhanced production and proliferation of myelomonocytic precursor cells in the haematopoietic organs, as well as the accumulation of monocyte/macrophages in tissues of the mutant mice (Shultz *et al*. 1993; Mlinaric-Rascan *et al*. 1994), although a role for inflammatory stimuli cannot be ruled out after 3 weeks of age.

In contrast to the accumulation of monocyte/macrophages in many organs and tissues, the development of marginal metallophilic macrophages and marginal zone macrophages was poor and their differentiation and maturation were impaired in the spleen of *me*^{v} mice. These abnormalities were accompanied by poor development of lymphoid follicles and marked reductions in the number of B and T cells in the splenic white pulp. In normal littermates, the development of both marginal metallophilic and marginal zone macrophage populations was incomplete at birth, but these cell populations developed during postnatal period with the development of B and T cells in the splenic pulp and encircled the marginal zone completely by 3 weeks after birth. Previous elimination and repopulation studies of marginal metallophilic and marginal zone macrophage populations by a single injection of liposomes containing dichloromethylene diphosphonate demonstrated a close

Figure 5. Immunohistochemical detection of Ly-1- and F4/80-positive macrophages in the peritoneal cavity (a), spleen (b), bone marrow (c), lymph node (d), and omentum (e, f), as well as Ly-1- and ER-MP20- or ER-MP58-positive precursor cells (g, h) in milky spots of omentum in *me*^{v} mice. Arrows indicate Ly-1-positive macrophages on double immunostaining with Ly-1 (blue) and F4/80 (brown) (a-f). Double positive cell for Ly-1 (blue) and ER-MP20 (brown) (g) and for Ly-1 (blue) and ER-MP58 (brown) (h). × 50 (e) and × 500 (a-d, f-h)

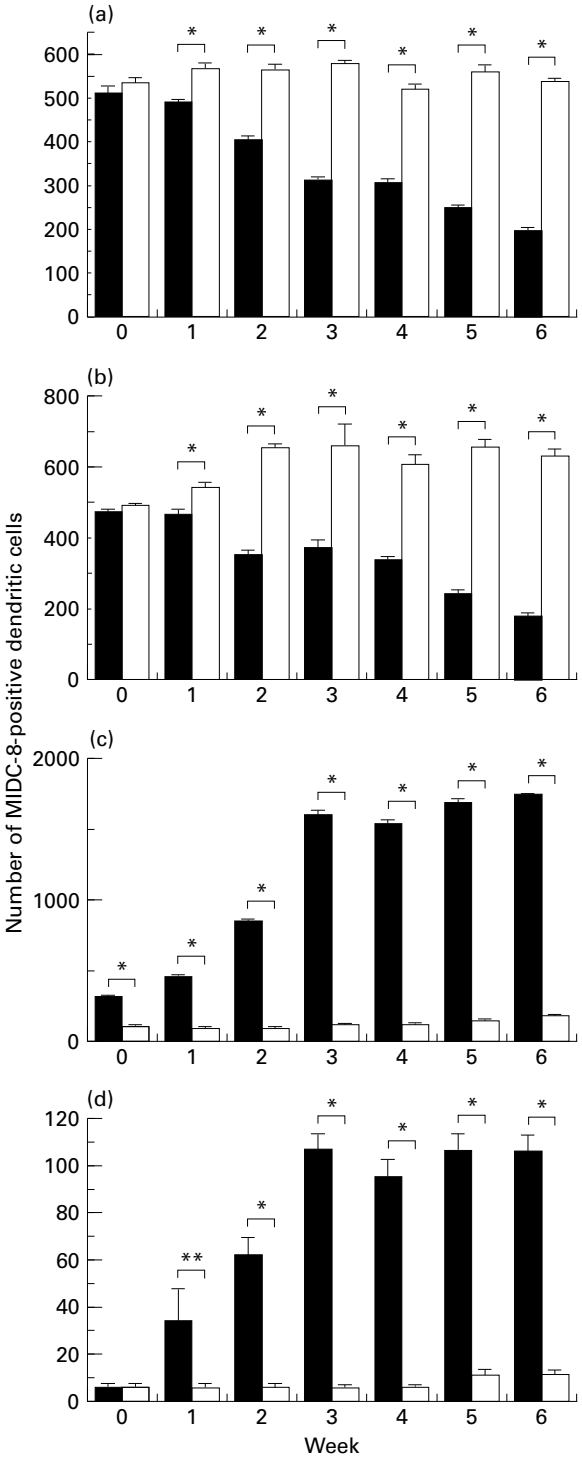

Figure 6. Numbers of MIDC-8-positive dendritic cells in (a) the spleen, (b) the thymus, (c) the lungs and (d) the liver of *me*^{v} mice (■) and normal littermates (+/–)(□) at 4 days after birth (week 0) and at the age 1–6 weeks (*$P<0.01$,**$P<0.05$). Cell numbers ± SD are representative of at least 3 mice of each type examined at each week

relationship between the repopulation of both macrophage subpopulations and the localization of B cells in the marginal zone (Claassen *et al.* 1986; Kraal *et al.* 1989). Taking all this information and our data together, it may be speculated that the poor development of MOMA-1- and ER-TR9-positive macrophage populations in *me*^{v} mice is associated with microenvironmental abnormalities and dysorganization of lymphoid tissues, particularly the marginal zone.

In contrast with severe impairments in the development and differentiation of B-2 cells, *me* and *me*^{v} mice are known to have enhanced production of Ly-1 B (B-1) cells, their differentiation into plasma cells, and production of Ig and autoantibodies (Shultz *et al.* 1987; Schweitzer & Shultz 1988; Schweitzer *et al.* 1991; Sidman *et al.* 1984, 1986). A previous study showed that high numbers of B-1 cells develop in the peritoneal cavity and spleen of *me*^{v} mice and that peritoneal B-1 cells expressed a high density of sIgM, Mac-1, Ly-1, and Pgp-1 and showed enhanced responses to phorbol ester (Kolber *et al.* 1991). More recently, Borrello & Phipps 1996) reported that Ly-1-positive macrophages were present in the spleen of *me*^{v} mice. In the present study, flow cytometry and immunohistochemical double staining demonstrated the presence of Ly-1-positive macrophages as well as of B-1 cells in the peritoneal cavity, spleen, and bone marrow of the mutant mice. The percentages of Ly-1-positive macrophages in the peritoneal cavity were obviously higher than those in the spleen and bone marrow of the mutant mice. In addition to the presence of abundant Ly-1-positive macrophages, the present study demonstrated in the peritoneal cavity of *me*^{v} mice that there were numerous B220-positive macrophages, B220- and ER-MP20-positive precursor cells, and a minor population of Ly-1- and ER-TR20-positive precursor cells. We also demonstrated an increased percentage of Ly-1-positive B cells (B-1 cells) in the peritoneal cavity of *me*^{v} mice, compared to normal littermates. In the foetal and adult stages of mice and humans, Ly-1-positive B cells are known to develop in the omentum, particularly within the milky spots (Solvason *et al.* 1992). In the present study, we detected the presence of Ly-1-positive macrophages and their precursor cells in milky spots of the omentum in *me*^{v} mice, suggesting that these macrophages are derived from single common precursor cells for both B-1 cells and macrophages in the peritoneal cavity of *me*^{v} mice, particularly in the omental milky spots.

Dendritic cells are heterogeneous populations which normally function in antigen presentation to T or B cells and are classified into two major populations (Fossum 1989). In both major populations, epidermal Langerhans

cells, veiled cells in dermis and lymph, and interdigitating cells in the T cell-dependent area of lymph nodes or the other peripheral lymphoid tissues express major histocompatibility complex class II antigens (Ia antigens), function as antigen presenting cells to T cells, and induce mixed leukocytic reaction (Fossum 1989) and are called T cell-associated dendritic cells (Tew 1992). Recently, it has been proposed that there are two distinct lineages of dendritic cell differentiation: one is a specific cell lineage from dendritic cell colony-forming unit (CFU-DC) or from haematopoietic stem cells to dendritic cells (Langerhans cells in skin or interdigitating cells in lymphoid tissues) and the other from monocytes to dendritic cells (Caux *et al*. 1994, 1995; Takahashi *et al*. 1996; Rescigno *et al*. 1997). In *me^v* mice, in addition to severe numerical reduction of epidermal Langerhans cells in skin (Shultz 1987, 1988), the present study demonstrated that the numbers of interdigitating dendritic cells in thymic medulla, lymph nodes, and peripheral lymphoid tissues were reduced with age. In these tissues, the dendritic cells were positive for NLDC-145 and/or MIDC-8 but not for ER-MP20. In contrast, the numbers of dendritic cells were increased in lungs and liver. In these tissues, MIDC-8- and ER-MP20-double positive dendritic cells were occasionally detected, suggesting that these dendritic cells are derived from monocytic cells markedly expanded in *me^v* mice. Such numerical reductions of Langerhans/dendritic cells in the skin, thymic medulla, and peripheral lymphoid tissues, and marked infiltration of dendritic cells in lungs and liver of *me^v* might reflect differences of microenvironments for the development and kinetics of dendritic cell populations between the epidermal or lymphoid tissues and the lungs or liver.

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