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Effects of Granulocyte/Macrophage Colony-Stimulating Factor on the Development and Differentiation of CD5-Positive Macrophages and Their Potential Derivation from a CD5-Positive B-Cell Lineage in Mice

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In co-cultures of either the murine pre-B cell line J13, fetal liver cells, or adult peritoneal or bone marrow cells with ST2 mouse bone marrow stromal cells in the presence of granulocyte/macrophage colony-stimulating factor (GM-CSF), the development of CD5$^+$ macrophages was demonstrated by immunohistochemical staining and flow cytometry. Although CD5$^+$ macrophages were not present in the peritoneal cavities of normal mice, approximately 30% of the peritoneal macrophages in viable motheaten (*me*^{v}/*me*^{v}) mice, deficient in SHP-1 protein tyrosine phosphatase, expressed cell surface CD5 and B220, markers for B cells. In the *me*^{v}/*me*^{v} mice, GM-CSF level in peritoneal fluid was increased significantly. At 5 days after daily intravenous injection with GM-CSF, many CD5$^+$ macrophages appeared in the peritoneal cavity and in omental milky spots of normal mice but fewer in osteopetrosis (*op*) mutant mice, deficient in macrophage (M)-CSF. These results indicate that GM-CSF, in combination with M-CSF, induces the development and differentiation of CD5$^+$ macrophages in the peritoneal cavity, particularly in the omental milky spots of mice. In the peritoneal cavity of GM-CSF-treated mice, the percentages of hematopoietic progenitor cells doubly positive for CD5 and CD34 or c-kit and of macrophage precursor cells doubly positive for CD5 and ER-MP58 or ER-MP20 were increased significantly during the development of CD5$^+$ macrophages and CD5 B cells, suggesting that CD5$^+$ macrophages and B cells may share a bipotential progenitor *in vivo*. *(Am J Pathol 1998, 152:445–456)*

A lymphocytic origin of macrophages was proposed by Maximow and Bloom early this century,[1,2] a view supported by several investigators on the basis of their ultrastructural or experimental studies.[3–7] *In vitro* studies of cell clones established from B-cell lymphomas[8,9] or normal murine B cells[10–12] demonstrated certain characteristics of macrophages,[8–12] whereas immunoglobulin (Ig) gene rearrangements have been detected in human cases of acute myelogenous leukemia.[13] Furthermore, cloned pre-B and B cell lines from bone marrow cells in Eu-*myc* transgenic mice infected with a retrovirus bearing v-*raf* developed into macrophages. These cells retained parental Ig gene rearrangements,[14] suggesting a switching of B-cell lineage into myeloid cells. Murine bone marrow cells infected with replication-defective retroviruses containing v-*raf* alone or v-*myc* alone yielded transformed pre-B cell lines, whereas a retroviral construct of both v-*raf* and v-*myc* oncogenes produced clonally related populations of mature B cells and macrophages.[15] Expression of the M-CSF receptor has been reported in the switching of pre-B cell lines into macrophages *in vitro*.[16]

Two distinct B-cell lineages exist: conventional B cells and Ly-1 (CD5) B cells (B-1 cells).[17] B-1 cells are further classified phenotypically into two subsets: B-1a (CD5$^+$) and B-1b (sister, CD5$^-$).[18,19] Evidence for a developmental relationship between CD5$^+$ B-lineage cells and macrophages has been reported in *in vitro* studies.[20–22] In a study of eight different Ly-1-positive (CD5$^+$) pre-B cell lines established in a long-term bone marrow culture

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system and showing a germ-line configuration of the joining (J) region segments of the Ig heavy-chain genes, Katoh et al[20] reported that three of the cell lines showed differentiation into Ly-1 B cells in co-culture with a mouse bone marrow stromal cell line, ST2, in the presence of interleukin (IL)-5, whereas one, co-cultured with ST2 and granulocyte/macrophage colony-stimulating factor (GM-CSF), acquired Mac-1 expression, retained Ly-1 expression, and developed into macrophages morphologically, cytochemically, and functionally. Similar differentiation was demonstrated in *ras*-transformed pre-B cell lines.[21,22] Cumano et al[23] purified and characterized mouse fetal liver cells, generated clones containing both macrophages and B cells in a culture, and showed the common origin of the two cell types from single precursor cells. *In vivo*, Borrello and Phipps[24] detected $CD5^+$ macrophages in the spleen of motheaten (*me/me*) mice. In our recent study, we found that $CD5^+$ macrophages were more numerous in the peritoneal cavity than the spleen and other tissues of viable motheaten (me^v/me^v) mice and responded to GM-CSF.[25] However, little is known about the development and differentiation of $CD5^+$ macrophages *in vivo*.

The aim of the present study was to examine effects of GM-CSF on the development and differentiation of $CD5^+$ macrophages *in vitro* and *in vivo*. First, we examined the morphological, immunohistochemical, and ultrastructural changes of a $CD5^+$ pre-B cell line established by Katoh et al[20] into $CD5^+$ macrophages after its co-culture with ST2 and GM-CSF. Second, we studied the emergence of $CD5^+$ macrophages in the co-culture of mouse fetal liver cells, adult mouse bone marrow cells, and peritoneal wash cells with ST2 in the presence or absence of GM-CSF by immunohistochemistry and flow cytometry using anti-mouse monoclonal antibodies for macrophages and B cells. In various tissues of adult normal or mutant mice, including me^v/me^v mice, $CD5^+$ macrophages were detected immunohistochemically, immunoelectron microscopically, or by flow cytometry. Finally, we examined the effects of GM-CSF on the development and differentiation of $CD5^+$ macrophages and changes of their hematopoietic progenitor cells and macrophage precursor cells in the peritoneal cavity of normal or mutant mice.

Materials and Methods

Animals

BALB/c Slc mice and C3H/He Slc mice were purchased from Nihon SLC Co. (Hamamatsu, Japan) and C3H/HeN and C.B-17/Icr-*scid* Jcl mice from Nihon Clea Co. (Tokyo, Japan). Nude mice $Hfhl^{nu}/Hfhl^{nu}$ (*nu/nu*), Lasat mice, C57BL/6 mice, BXSB/MpJ-*Yaa* mice, MRL/MpJ Fas^{lpr}/Fas^{lpr} (*-lpr/lpr*) mice, and NZBW/F1 mice were also purchased from Nihon SLC Co. All of these mice were examined at approximately 10 weeks of age. Fetal livers were obtained from 15- or 18-day-old fetuses of BALB/c mice. Breeders of viable motheaten (me^v/me^v) mice and osteopetrosis (*op/op*) mice were supplied from the Jackson Laboratory (Bar Harbor, ME). C57BL/6J-$Hcph^{me-v}/Hcph^{me-v}$ (me^v/me^v) mice and (C3HeBFeJ × C57BL/6J)-$csfm^{op}/csfm^{op}$ (*op/op*) mice and their normal littermates (+/?) were obtained by mating of $+/me^v$ breeders or +/*op* heterozygotes, respectively, and examined at 3 or 4 weeks after birth. Me^v/me^v mice were distinguished from normal littermates (+/?), because of small alopecic lesions in the head. Homozygous *op/op* mice were distinguished from the normal littermates (+/?) because the postnatal development of *op/op* mice delays and because they develop a small body, short tail, round skull, and defect of incisors at approximately 10 days after birth. In addition, by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis, mutant mice (−/−), heterozygotes (+/−), and wild-type mice (+/+) were discriminated, and the mutant and wild-type mice were used for examination.

Tissue and Free Cell Preparation

Bone marrow, liver, and spleen were removed from adult BALB/c mice for immunohistochemistry. One-half of these tissues were minced, mashed, and filtered through nylon mesh to prepare free cell suspensions. For culture, these procedures were performed under aseptic conditions, and bone marrow cells were passed twice through a G-10 column to remove pre-existing macrophages.

By washing with phosphate-buffered saline (PBS), peritoneal cells were collected from unmanipulated adult mice or from three adult mice 30 minutes after intraperitoneal injection of 0.2 ml of latex beads (0.81 μm in diameter; Sigma Chemical Co., St. Louis, MO). To collect alveolar cells, bronchoalveolar lavage was carried out on adult BALB/c mice according to the method described previously.[26]

Preparation of GM-CSF from the Supernatant of a GM-CSF-Producing Mouse Melanoma Cell Line

A GM-CSF-producing mouse melanoma cell line (B16 cells),[27] kindly given by Dr. Hirofumi Hamada (National Cancer Institute, Tokyo, Japan), was grown into confluence in RPMI medium containing 10% (v/v) fetal calf serum. After culture with serum-free medium, the supernatant was obtained from the cultures, dialyzed, and condensed for use. Recombinant murine GM-CSF was purchased from Genzyme (Cambridge, MA). To determine the dose of GM-CSF for administration, BALB/c or C3H/He mice were injected subcutaneously with 1, 5, or 10 ng of GM-CSF daily for 5 days, and the numbers of $F4/80^+$ macrophages in the liver were counted and compared. As a result, 5 ng of GM-CSF was the most effective cytokine dose for numerical increment of macrophages. And also, we found no significant difference in macrophage increase between recombinant murine GM-CSF and condensed GM-CSF prepared from the culture supernatant in the culture of the GM-CSF-producing mouse melanoma cell line.

Table 1. Antigen Specifities and Immunoreactivity of Monoclonal Antibodies Used for Immunohistochemistry and/or Flow Cytometry

Monoclonal antibodies	Immunoreactive cells or substances
F4/80	Promonocytes, monocytes, free or fixed macrophages, Kupffer cells, histiocytes, synovial A cells, microglia, phagocytes on the peri- and endosteal surfaces, and epidermal Langerhans cells
BM8	Tissue-fixed macrophages and epidermal Langerhans cells
ER-MP 20	Colony-forming unit-macrophage, monoblasts, promonocytes, monocytes, and immature macrophages
ER-MP 58	Myeloid precursor cells (M-CSF-responsive colony-forming unit-granulocyte/macrophage)
Cfms	Receptor for M-CSF
CD34	Hematopoietic progenitor cells
c-kit	Transmembrane tyrosine kinase receptor for stem cell factor
CD5	Mature T cells and a small subset of B cells
B220	Pre-B and B lymphocytes
IgM	Heavy chain of IgM

Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of GM-CSF in the condensed supernatant of the above melanoma cell culture and in peritoneal fluid of mice was measured with a murine GM-CSF ELISA kit (Endogen, Boston, MA).

Culture and Cell Lines

RPMI 1640 medium and fetal bovine serum were purchased from Sigma and Microbiological Associates (Walkersville, MD), respectively. The J13 pre-B cell line was established previously,[20] which shows a germ line of J region segments of the immunoglobulin heavy chain (IgH) gene. The ST2 stromal cell line, established by Ogawa et al,[28] was kindly provided by Dr. S-I. Nishikawa (Kyoto University, Kyoto, Japan). The J13 cells were co-cultured on ST2-adherent stromal cells in the presence of macrophage (M)-CSF for 4 weeks according to procedures described previously.[20] Cell suspensions prepared from the fetal mouse liver cells, adult mouse bone marrow cells, and peritoneal cells as above were co-cultured on ST2 monolayers with changes of culture medium at 4-day intervals. The same co-culture, with 5 ng of recombinant murine GM-CSF added, was used with changes at 2-day intervals.

Monoclonal Antibodies

For immunohistochemistry, rat monoclonal antibodies against mouse macrophages F4/80 and BM8 (BMA Biomedical, August, Switzerland), those for macrophage precursor cells ER-MP58 or ER-MP20 (BMA Biomedical), a monoclonal antibody against mouse T cell differentiation antigen CD5 (Ly-1), a monoclonal antibody against IgM (Lo. MM-9, Serotec, Oxford, UK), and a monoclonal antibody for hematopoietic stem cell CD34 (Pharmingen, San Diego, CA) were used.

For double-immunofluorescence staining by flow cytometry, we used fluorescein isothiocyanate (FITC)- or biotin-conjugated anti-F4/80 (Serotec), FITC-conjugated anti-IgM (Lo. MM-9, Serotec), FITC- or biotin-conjugated anti-Ly-1 (CD5; Becton Dickinson, Mountain View, CA), B220 (CALTAG, San Francisco, CA), CD34, c-kit (Pharmingen), ER-MP20 (BMA Biomedical), biotin-conjugated anti-*Cfms* and ER-MP58, and phycoerythrin-conjugated streptavidin (Serotec). Biotinylated anti-*Cfms* and ER-MP58 were kindly supplied by Dr. S-I. Nishikawa (Kyoto University) and Dr. P. J. M. Leenen (Erasmus University, Rotterdam, The Netherlands), respectively. Table 1 shows antigen specificities and immunoreactivity of the monoclonal antibodies used for the present immunohistochemical double staining and flow cytometry.[20,29–37]

Light Microscopy

Cytospin preparations of cultured cells were stained with May-Gruenwald-Giemsa solution (Merck, Darmstadt, Germany).

Immunohistochemistry

To detect $CD5^+$ and $F4/80^+$ or $BM8^+$ cells in tissues, immunohistochemical double staining was performed as described previously.[16] In brief, the tissues obtained above were fixed in 2% periodate-lysine-paraformaldehyde fixative at 4°C for 4 hours. After washes with PBS containing 10, 15, and 20% sucrose and OCT compound (Miles Laboratories, Elkhart, IN), the tissues were frozen in dry ice/acetone and cut with a cryostat (Bright, Hitington, UK) into 6-μm-thick sections. The other tissues were frozen by slowly dipping them into liquid nitrogen, embedded in OCT compound, and cut into cryostat sections. These sections were fixed further with acetone for 10 minutes. A pair of cryostat sections were prepared and incubated with rat anti-mouse Fc receptor blocking antibody anti-CD16/CD32 (Pharmingen) for 5 minutes to exclude the contribution of Fc receptors. After inhibition of endogenous peroxidase activity according to the procedure of Isobe et al,[38] one section was incubated with one of the monoclonal antibodies for 60 minutes. As the second step, peroxidase-conjugated sheep anti-mouse Ig ($F(ab')_2$) diluted 1:100 (Amersham, Poole, UK) was reacted for 60 minutes. After staining this reaction brown with 3,3′-diaminobenzidine, the section was treated with 0.1 mol/L glycine/HCl buffer (pH 2.2) for 60 minutes with four changes to remove the first and second antibodies reacted. The same section was incubated overnight with one of the other monoclonal antibodies. After washes with

Tris-buffered saline (pH 7.6), the section was treated with alkaline-phosphatase-labeled rabbit anti-rat IgG antibody. To visualize the reaction for alkaline phosphatase, the section was stained blue in a substrate consisting of 0.2 mmol/L naphthol AS-MX phosphate, 1 mmol/L fast blue BB salt, and 1 mmol/L levamisole in 50 mmol/L Tris/HCl buffer (pH 8.9) for 20 minutes. Omitting the first step of the staining, another section was stained by the alkaline phosphatase method using the second monoclonal antibody alone. Substituting the first monoclonal antibody for the second one, the same double staining procedures were performed on another pair of sections at the same time.

Similar procedures of immunohistochemical double staining were performed on cytospin preparations of free cell suspensions or cultured cells after fixation with acetone at 4°C for 10 minutes. In the cytospin preparation of free cell suspensions, the method of Isobe et al[38] was omitted.

Electron Microscopy and Immunoelectron Microscopy

After 1 month of culture with ST2 and GM-CSF, J13 cells were subjected to the method of electron microscopy as below. Cytospin preparations of peritoneal cells collected from GM-CSF-treated BALB/c mice or me^v/me^v mice without or after intraperitoneal injection of latex particles were immunostained with anti-CD5 or anti-B220 as described above. The preparations were post-fixed with 1% osmium tetroxide in 0.05 mol/L cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanols, and embedded in Epon 812. Ultrathin sections were cut by Ultrotome Nova (LKB, Uppsala, Sweden) and observed in a JEM 2000EX (JOEL, Tokyo, Japan) after staining with lead citrate. Control slides were incubated with nonimmunized mouse serum or PBS instead of the primary antibody and then processed by the same procedure described above; the control stainings were invariably negative.

Immunofluorescence Staining and Flow Cytometry

The expression of cell-surface antigen was analyzed by flow cytometry as described previously.[34,39,40] Cells (1×10^6) were incubated with rat IgG2aκ and IgG2bκ for blocking nonspecific binding to any anti-mouse rat monoclonal antibodies, because all of the antibodies used in this study were rat IgG2aκ and IgG2bκ. After two washes, the cells were incubated with the Fc receptor blocking antibody anti-CD16/CD32 (Pharmingen) for 20 minutes to block Fc receptors. After two washes, the cells were incubated with biotinylated monoclonal antibodies for 20 minutes. After two or more washes, the cells were incubated with phycoerythrin-conjugated streptavidin and FITC-conjugated monoclonal antibodies for 20 minutes. For analysis, FACScan (Becton Dickinson) was used, and only the cells within a leukocyte gate were counted as described.[39,40]

Statistics

Statistical significance of the data was evaluated by Student *t*-test. P values of <0.05 were considered significant.

Results

Morphological, Immunophenotypic, and Ultrastructural Characterization of Macrophage-Like Cells Generated from J13 Cell Line after Long-Term Co-Culture with ST-2 Stromal Cells in the Presence of GM-CSF

Immunohistochemically, the J13 cell line was weakly positive for CD5 but negative for F4/80, BM8, ER-MP20, or ER-MP58. When co-cultured with ST2 stromal cells in the presence of GM-CSF for 4 weeks, the cell line showed macrophage-like morphology, marked phagocytosis of latex beads (Figure 1A), and were doubly positive for CD5 and F4/80 (Figure 1B), ER-MP20 (Figure C), or ER-MP58 (data not shown). Electron microscopy of the cultured cells showed immature macrophages with abundant polyribosomes and poorly developed intracellular organelles. These cells possessed phagocytic vacuoles and extended long cytoplasmic projections (Figure 2A).

Emergence of $CD5^+$ Macrophages in Co-Cultures of Fetal Mouse Liver Cells, Adult Mouse Peritoneal Cells, and Bone Marrow Cells on ST2 Monolayers in the Presence or Absence of GM-CSF

In co-cultures of liver cell suspensions from mouse fetuses at 15 and 18 days of gestation on ST2 monolayers, $CD5^+$ $F4/80^+$ cells were detected immunohistochemically from 13 to 21 culture days. In the similar co-cultures of adult mouse peritoneal cells or bone marrow cells, $CD5^+$ $F4/80^+$ cells were also demonstrated after 2 to 3 weeks of culture (Figure 1D) and showed the ultrastructure of a mature macrophage (Figure 2B). In this period, IgM-bearing $F4/80^+$ cells were also demonstrated. From 1 to 2 weeks, $CD5^+$ $ER\text{-}MP20^+$ or $ER\text{-}MP58^+$ cells were detected immunohistochemically in co-cultures of cell suspensions prepared from fetal mouse liver cells, adult mouse bone marrow cells, and peritoneal cells with ST2 (Figure 1E). In co-cultures of mouse peritoneal cells with ST2 and GM-CSF, $B220^+$ $F4/80^+$ macrophages were detected at 10 days of culture (Figure 1F).

By flow cytometric analysis, $CD5^+$ macrophages were not detected in the free cell suspensions prepared from the peritoneal wash cells and bone marrow cells of adult mice (Figure 3, A and B) and from fetal mouse livers. In the co-cultures of liver cell suspensions from 15- or 18-day-old mouse fetuses and of adult mouse peritoneal wash cells or bone marrow cells on ST2 monolayers, the numbers of $CD5^+$ $F4/80^+$ cells were slightly increased from 15 to 20 culture days. However, their percentages

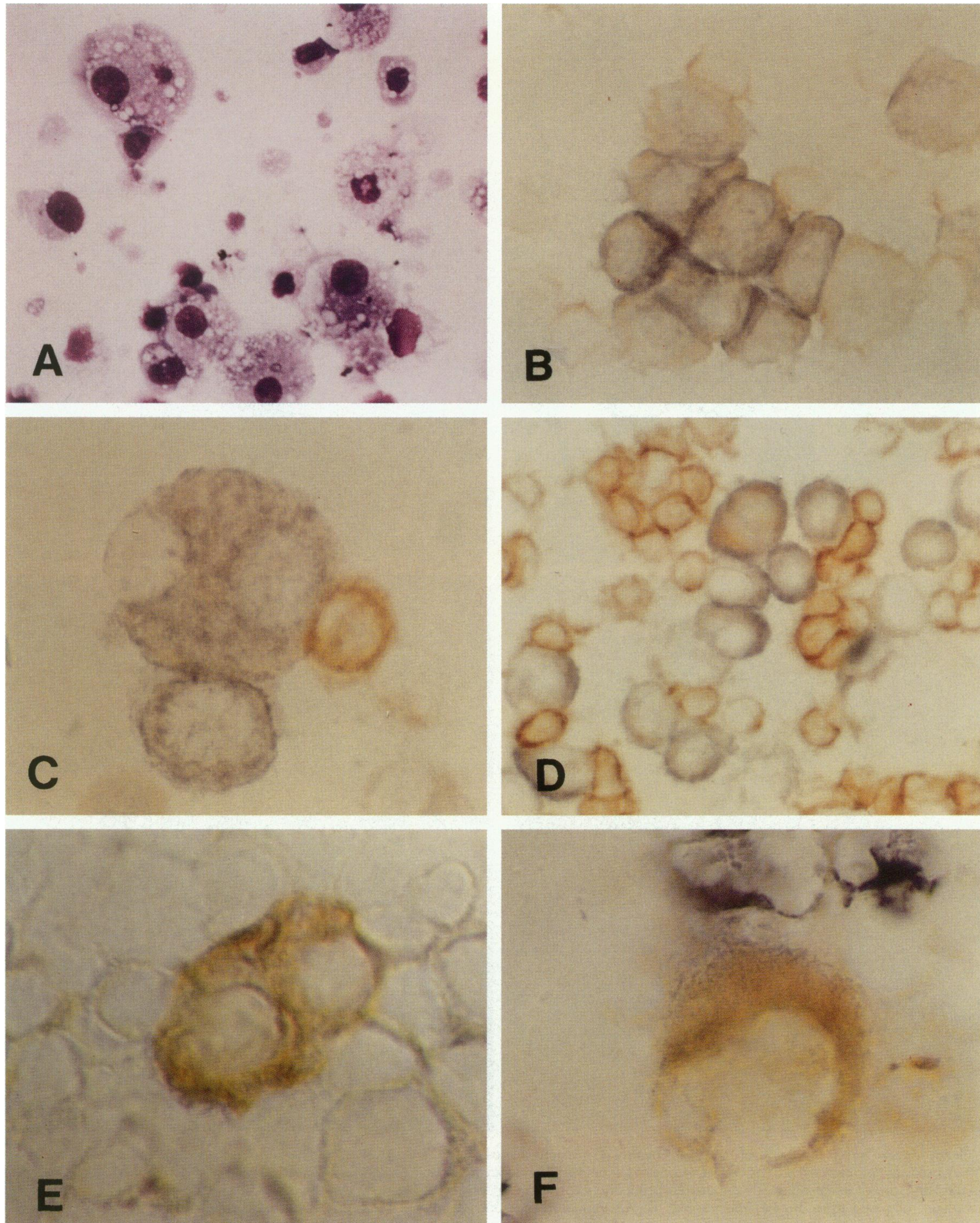

Figure 1. Co-cultures of J13 cells (**A** to **C**) or peritoneal wash cells of adult BALB/c mice (**D** to **F**) with ST2 stromal cells in the presence of GM-CSF. **A** to **C**: After 1 month, J13 cells show marked phagocytosis of latex beads (**A**) and are doubly positive for CD5 and F4/80 (**B**) or ER-MP20 (**C**). **D** to **F**: In 10-day co-culture of ST2 cells with adult mouse peritoneal cells, $CD5^+$ $F4/80^+$ cells (**D**), $B220^+$ $F4/80^+$ cells (**E**), and $CD5^+$ $ER\text{-}MP20^+$ cells (**F**) are found and stained grayish blue-brown. May-Gruenwald-Giemsa staining (**A**); immunohistochemical double staining with CD5 (**B** to **D** and **F**) or B220 (**E**) and F4/80 (**B**, **D**, and **E**) or ER-MP20 (**C** and **F**); magnification, ×400 (**A**, **B**, and **D**), ×1000 (**C**), and ×850 (**E** and **F**).

were below 5% of the total nonadherent culture cells. In the co-cultures of bone marrow cells or peritoneal wash cells on ST2 monolayers with GM-CSF, $CD5^+$ $F4/80^+$ cells appeared at 10 days of culture and were 87.8% and 52.7% of total cells, respectively (Figure 3, C and E). In the same co-cultures, the percentages of $B220^+$ $F4/80^+$ cells were 50.10% (Figure 3F). In the co-cultures of the bone marrow cells with ST2 and GM-CSF, $CD5^+$ $Cfms^+$ cells (Figure 3D) and $CD5^+$ IgM^+ cells were 46.58% and 70.24% of the total cells, respectively. However, the

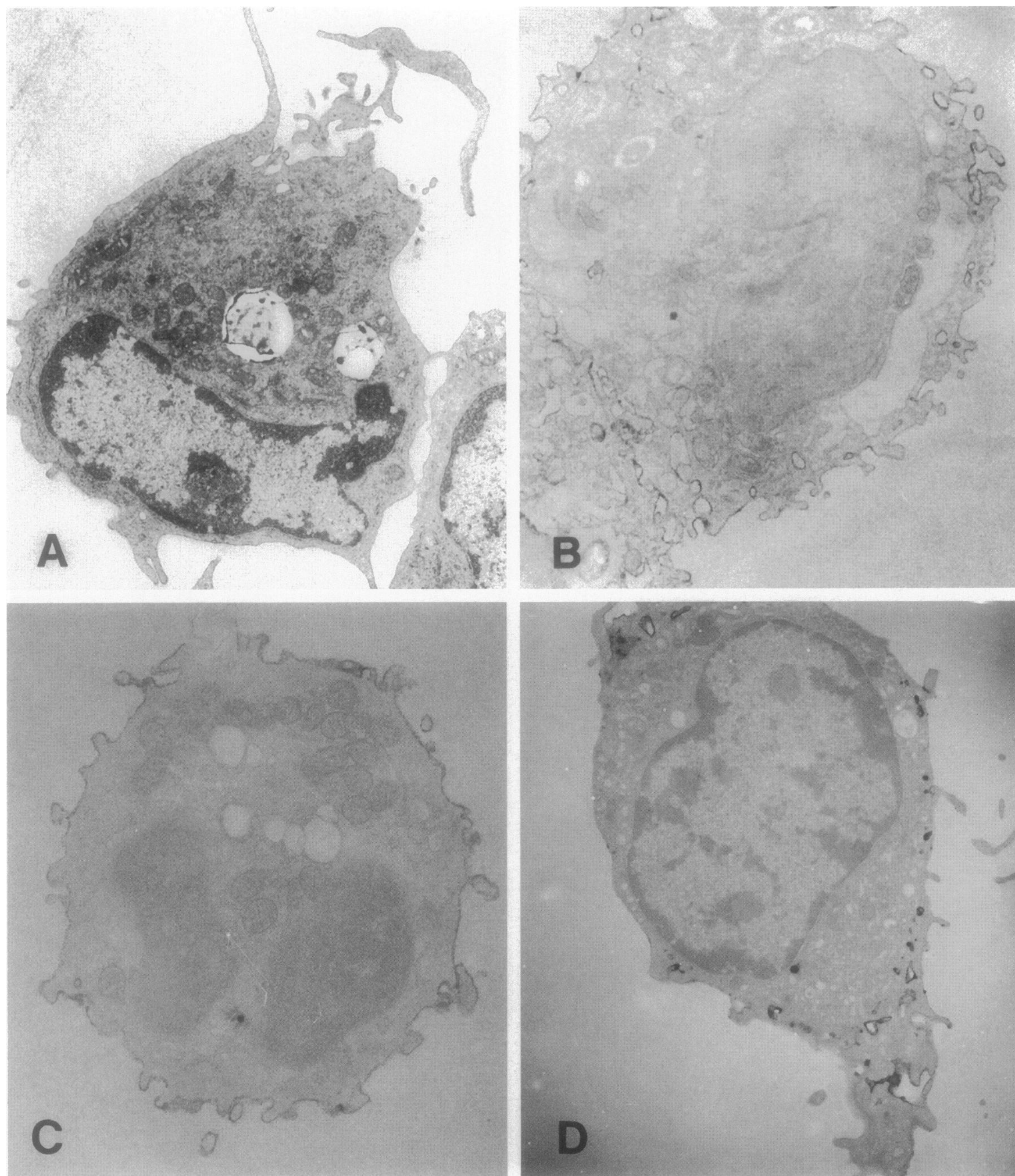

Figure 2. Electron micrograph of a cultured J13 cell (**A**) and CD5$^+$ macrophages (**B** to **D**). **A**: The J13 cell has an elongated, heterochromatic nucleus, projects a few short cytoplasmic processes from the cell surface, and contains two phagocytic vacuoles with loosely arranged, lamellar profiles. **B**: Cultured peritoneal cell shows the ultrastructure of macrophages, extends microvilli, and expresses CD5 on the cell surface membrane. **C**: CD5 is expressed on the surface membrane of a macrophage obtained from an *me^v/me^v* mouse. **D**: An immature macrophage in the peritoneal cavity of BALB/c mouse at 5 days of daily GM-CSF administration expresses CD5 on the cell membrane, particularly in coated pits. Electron microscopy (**A**); immunoelectron microscopy with CD5 (**B** to **D**); Magnification, ×3000 (**A**), ×5000 (**B**), and ×4500 (**C** and **D**).

emergence of CD5$^+$ F4/80$^+$ cells was extremely slight (less than 1%) in the co-cultures of 15- or 18-day-old mouse fetal liver cell suspensions with ST2 and GM-CSF at 7 days of culture. These results indicate that GM-CSF plays an important role in the development and differentiation of CD5 B-lineage cells into CD5$^+$ macrophages in the bone marrow and peritoneal cavity of adult mice.

In Vivo *Detection and Characterization of CD5$^+$ Macrophages in Adult Mice*

Flow cytometric analysis demonstrated that CD5$^+$ F4/80$^+$ cells were absent or extremely minor (less than 0.04%) in the peritoneal cavity of normal or immunodeficient mice of various strains (Table 2). In addition, CD5$^+$

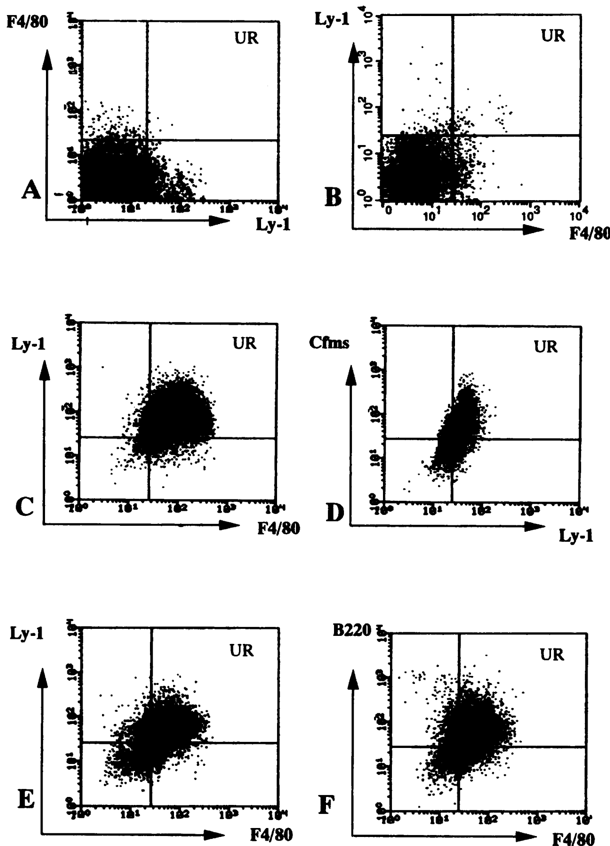

Figure 3. Flow cytometric analysis of CD5$^+$ macrophages in the free cell suspensions prepared from peritoneal wash cells and bone marrow cells of adult BALB/c mice (**A** and **B**) and in the co-culture of the bone marrow cells (**C** and **D**) and peritoneal wash cells (**E** and **F**) on ST2 cell line monolayers in the presence of GM-CSF at 10 days of culture. **A:** Lack of CD5$^+$ F4/80$^+$ cells in the peritoneal cell suspension. **B:** Lack of CD5$^+$ F4/80$^+$ cells in the bone marrow cell suspension. **C:** CD5$^+$ F4/80$^+$ cells in the co-culture of bone marrow cells. **D:** CD5$^+$ *Cfms*$^+$ cells in the co-culture of bone marrow cells. **E:** CD5$^+$ F4/80$^+$ cells in the co-culture of peritoneal wash cells. **F:** B220$^+$ F4/80 $^+$ cells in the co-culture of peritoneal wash cells. All of these doubly positive cells are shown in the upper right square (UR) of each figure.

Table 2. Flow Cytometric Analysis of CD5$^+$ Peritoneal Macrophages in the Adult Mice of Various Strains

Strain	CD5$^+$ F4/80$^+$ macrophages (%) ± SD*
BALB/c Slc	0.03 ± 0.01
C3H/He Slc	0.03 ± 0.01
C3H/HeN	0.07 ± 0.002
C57BL/6	0.06 ± 0.01
NZBW/F1	0.11 ± 0.062
MRL/MpJ-lpr/lpr	0.07 ± 0.026
BXSB/MpJ-Yaa†	0.10 ± 0.056
Lasat	0.11 ± 0.036
nude (nu/nu)	0.28 ± 0.139
scid	0.33 ± 0.199
op/op‡	0.03 ± 0.01
me^v/me^v‡	36.29 ± 4.08
me^v littermates (+/+)	0.02 ± 0.01

*Percentage of CD5$^+$ F4/80$^+$ macrophages in the total peritoneal wash cells.
†More than three animals were examined at approximately 10 weeks of age.
‡Examined at 3 or 4 weeks of age.

F4/80$^+$ cells were not detected in bone marrow cells, spleen cells, or bronchoalveolar lavage cells of these mice. Immunohistochemically, CD5$^+$ F4/80$^+$ cells were also undetectable in the bone marrow, spleen, and other tissues of the mice. In me^v/me^v mice, more than 30% of the peritoneal cells were CD5$^+$ F4/80$^+$ in the peritoneal cavity (Table 2) and revealed the ultrastructure of macrophages (Figure 2C). CD5$^+$ F4/80$^+$ cells were also detected in the bone marrow and spleen of me^v/me^v mice, although their percentages were less than 5%. In the wild-type me^v littermates (+/+), there were no peritoneal macrophages doubly positive for CD5 and F4/80 (Table 2). On immunohistochemical analysis, many peritoneal macrophages in me^v/me^v mice revealed double-positive staining for CD5 and F4/80 or BM8. Compared with the CD5$^+$ macrophages, however, B220$^+$ F4/80$^+$ cells were mostly round and smaller in the mutant mice. By immunohistochemical double staining, many CD5$^+$ F4/80$^+$ macrophages were detected in the milky spots, and they were scattered around milky spots and in the omentum of the me^v/me^v mice (Figure 4B). However, there were few in the normal wild-type me^v littermates, as in BALB/c mice (Figure 4A). ELISA showed significant increases of GM-CSF levels in the peritoneal fluid of me^v/me^v mice ($P < 0.05$) compared with that of the normal wild-type me^v littermates (Table 3).

Development and Differentiation of CD5$^+$ Macrophages in the Peritoneal Cavity of Mice after Daily GM-CSF Administration

By flow cytometric analysis, CD5$^+$ F4/80$^+$ cells were absent in the peritoneal wash cells of BALB/c and C3H/He mice, as well as in the wild-type me^v or *op* littermates (+/+) (Table 4). To examine whether or not GM-CSF induces the development and differentiation of CD5$^+$ macrophages *in vivo*, all of these mice were injected subcutaneously with 5 ng of purified or recombinant murine GM-CSF every day and killed at 5 days after daily injection. Flow cytometric analysis showed significantly increased percentages of CD5$^+$ F4/80$^+$ macrophages in the peritoneal wash cells of all of these GM-CSF-treated mice including the wild-type *op* or me^v littermates ($P < 0.05$), compared with untreated mice (Table 4). In addition, we found the emergence of CD5$^+$ macrophages in the spleen and bone marrow of GM-CSF-treated mice; however, their percentages were low (less than 1.5%).

Immunoelectron microscopy revealed that positive reaction products for CD5 were localized on the cell surface membrane of peritoneal macrophages in GM-CSF-treated BALB/c mice (Figure 2D). The increased percentages of CD5$^+$ peritoneal macrophages in the GM-CSF-treated wild type me^v littermate mice were not over those of me^v/me^v mice. However, the increased percentages of CD5$^+$ peritoneal macrophages in GM-CSF-treated *op/op* mice were lower than those in GM-CSF-treated wild type *op* littermate mice (Table 4).

Table 5 shows the changes in percentage of CD5$^+$ hematopoietic progenitor cells, CD5$^+$ macrophage pre-

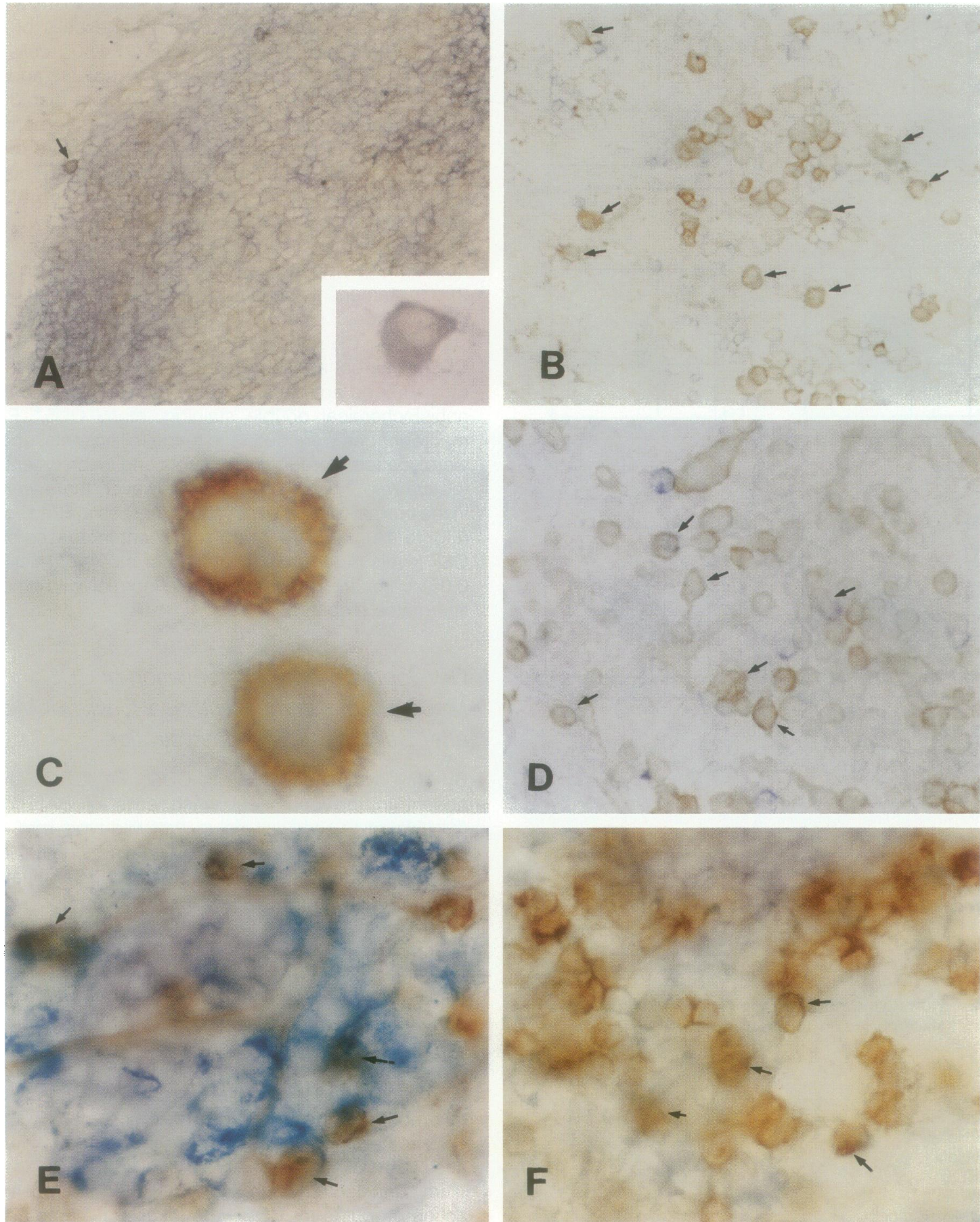

Figure 4. Immunohistochemical detection of $CD5^+$ macrophages and their precursors in the milky spots of BALB/c mice (**A**), *me^v/me^v* mice(**B** and **C**), and GM-CSF-treated BALB/c mice (**D** to **F**). **A:** In the milky spot of a BALB/c mouse, a few $CD5^+$ $F4/80^+$ cells are seen in its margin (**arrow**). **Inset:** A high magnification of the double-positive cell indicated by the **arrow**. **B:** Many $CD5^+$ $F4/80^+$ cells are found in the milky spot of the *me^v/me^v* mouse. **C:** A high magnification of two double-positive cells in the milky spot of the *me^v/me^v* mouse. Both cells stain grayish blue brown. **D** to **F:** Numerous $CD5^+$ $F4/80^+$ cells (**D**), $CD5^+$ ER-MP58 cells (**E**), and $CD5^+$ $CD34^+$ cells (**F**) are stained grayish blue brown (**arrows**) in the milky spot of a BALB/c mouse given GM-CSF for 5 days. Immunohistochemical double staining with Ly-1 and F4/80 (**A** to **D**), ER-MP58 (**E**), or CD34 (**F**); magnification, ×80 (**A**), ×600 (**inset**), ×100 (**B**), ×800 (**C**), ×200 (**D**), and ×450 (**E** and **F**).

cursor cells, $CD5^+$ macrophages, and CD5 B cells in the peritoneal cavity of BALB/c mice before and after daily GM-CSF administration. In the normal mice, CD5 and CD34 or c-kit double-positive cells ($CD5^+$ hematopoietic progenitor cells) were present in the peritoneal cavity, although their percentages were less than 5%. In addition, CD5 and B220 or IgM double-positive cells (B-1 cells) were also present. However, $CD5^+$ $F4/80^+$ macro-

Table 3. GM-CSF Levels in Peritoneal Fluid of Mice Measured by ELISA

Strain	GM-CSF (pg/ml)
BALB/c slc	ND
me^v/me^v (−/−)	26.7 ± 15.9
me^v littermates, Wild type (+/+)	ND

ND, not detectable.

phages were not detected. At 5 days after daily GM-CSF administration, $CD5^+$ macrophages appeared, accompanied by significant increases in percentage of $CD5^+$ hematopoietic progenitor cells, $CD5^+$ macrophage precursor cells, and CD5 B cells ($P < 0.01$). These data indicate that GM-CSF administration induces the development of $CD5^+$ macrophages and their differentiation from $CD5^+$ hematopoietic progenitor cells via $CD5^+$ macrophage precursor cells as well as the development of CD5 B cells, suggesting that there are common progenitor cells for $CD5^+$ macrophages and CD5 B cells.

Immunohistochemical double staining revealed increased numbers of $CD5^+$ $F4/80^+$ cells in the milky spots of omentum of the GM-CSF-treated BALB/c mice (Figure 4, C and D) compared with the untreated mice (Figure 4A). Similar development of $CD5^+$ macrophages in the milky spots was observed in GM-CSF-treated C3H/He mice as well as in GM-CSF-treated wild-type *op* or me^v littermate mice. Besides $CD5^+$ macrophages, $CD5^+$ $ER\text{-}MP20^+$ or $ER\text{-}MP58^+$ cells (Figure 4E) and $CD5^+$ $CD34^+$ cells (Figure 4F) were detected in the milky spots of the GM-CSF-treated mice.

Discussion

In a previous study, cells examined from the J13 cell line after 1 month of co-culture on an ST2 stromal cell monolayer in the presence of GM-CSF were reported to show macrophage-like morphology, phagocytosis of latex beads, positive histochemical reaction for nonspecific esterase, and immunophenotypic expression of Mac-1 and CD5.[20] By Southern blot analysis, the $CD5^+$ macrophages isolated from the co-cultures with ST2 stromal

Table 4. Flow Cytometric Analysis of $CD5^+$ Peritoneal Macrophages in the Adult Mice of Various Strains before or at 5 Days of Daily Subcutaneous Administration of Recombinant Murine GM-CSF

Strain	$CD5^+$ $F4/80^+$ macrophages (%) ± SD*	
	Day 0†	Day 5
BALB/c slc	0.0 ± 0.0	22.5 ± 2.5‡
C3H/He slc	0.0 ± 0.0	19.6 ± 0.9‡
op/op	0.0 ± 0.0	2.0 ± 0.3‡
op littermates, wild type (+/+)	0.0 ± 0.0	20.1 ± 2.3‡
me^v/me^v (−/−)	36.3 ± 4.1	36.4 ± 2.0
me^v littermates, wild type (+/+)	0.0 ± 0.0	25.3 ± 1.9‡

*Percentage of $CD5^+$ $F4/80^+$ macrophages in the total peritoneal wash cells.
†Examined before GM-CSF administration.
‡$P < 0.05$ by Student *t*-test.

Table 5. Changes in Percentage of $CD5^+$ Hematopoietic Progenitor Cells, $CD5^+$ Macrophage Precursor Cells, $CD5^+$ Macrophages, and CD5 B Cells before (Day 0) and at 5 Days after Daily GM-CSF Administration*

Cell type	Day 0 (% ± SD)	Day 5 (% ± SD)
CD5 F4/80	0.0 ± 0.0	22.5 ± 2.5*
CD5 *Cfms*	1.0 ± 0.8	18.5 ± 10.3*
CD5 B220	7.1 ± 3.3	22.7 ± 2.4*
CD5 IgM	10.1 ± 4.1	25.1 ± 5.7*
CD5 ER-MP20	0.4 ± 0.6	24.7 ± 0.8*
CD5 ER-MP58	11.7 ± 0.6	21.8 ± 6.9*
CD5 CD34	0.7 ± 1.2	20.0 ± 5.1*
CD5 c-*kit*	0.1 ± 0.3	26.7 ± 8.2*

Results are expressed as the percentage of double-positive cells in the total peritoneal wash cells. More than three mice were examined per group.
*$P < 0.01$ by Student *t*-test.

cells were shown to have the same configuration in the J region segments of IgM genes as their parental lymphoid cells and the same $J\kappa$ rearrangement.[20] Northern blot analysis revealed an abundant expression of $C\mu$, $\lambda5$, and *Cfms* mRNA in the J13 cell line.[20] In this study, we confirmed more definitely the cytological, immunophenotypic, and ultrastructural characteristics of macrophages in the converted J13 cells. Immunohistochemically, these cells expressed a doubly positive immunoreactivity for F4/80 and BM8 (anti-mouse monoclonal antibodies specific for macrophages) and for CD5 and B220 (monoclonal antibodies for CD5 B cells), and their ultrastructural features corresponded to those of immature macrophages. Together with the previous information, these data provide additional morphological evidence that J13 cells, a pre-B cell line, differentiate into $CD5^+$ macrophages in the co-culture with ST2 in the presence of M-CSF, suggesting a close developmental relationship between CD5 B-lineage cells and macrophages.

ST2 is a mouse bone marrow stromal cell line established by Ogawa et al,[28] which supports B lymphopoiesis and myeloid cell growth of not only adult mouse bone marrow cells but also fetal mouse liver hematopoietic cells.[28,41,42] It also supports the development and differentiation of these cells into macrophages.[43–46] To examine whether or not any $CD5^+$ macrophage progenitors are present in mouse tissues, we performed co-culture experiments on adult mouse peritoneal wash cells, bone marrow cells, and fetal mouse liver cells on an ST2 monolayer with or without GM-CSF to examine the emergence of $CD5^+$ macrophages by immunohistochemical double staining or flow cytometry. Although ST2 alone can induce $CD5^+$ macrophages in co-culture experiments, they appeared slowly and their percentages were low (less than 5%). By contrast, in the co-cultures with ST2 and GM-CSF, $CD5^+$ macrophages emerged earlier and more prominently and peaked at approximately 10 days of culture, supporting the view that GM-CSF in combination with M-CSF is important for the development and differentiation of $CD5^+$ macrophages *in vitro*, as reported in previous studies of B-cell lines.[16,20] These results provide evidence for the presence of $CD5^+$ $F4/80^+$ progen-

itor cells in hematopoietic cells of fetal mouse livers and in the bone marrow and peritoneal cavity of adult mice; they also suggest the existence of a differentiation pathway of CD5 B-lineage cells into macrophages.

CD5 B (B-1) cells are known to exist abundantly in the peritoneal cavity of adult mice, but they represent only a small fraction of the B cells in the spleen.[47] The present flow cytometric study did not demonstrate any CD5$^+$ F4/80$^+$ cells in any defined cell population in the peritoneal cavity of adult normal mice as reported in our previous preliminary study.[48] Recently, however, we could demonstrate the presence of numerous CD5$^+$ macrophages in the peritoneal cavity of viable motheaten (me^v/me^v) mice,[25] corresponding to the results of Borrello and Phipps[24] who reported the presence of CD5$^+$ macrophages in the spleen of *me/me* mice. The recessive *me* and me^v mutations on chromosome 6 disrupt the gene encoding a protein tyrosine phosphatase termed hematopoietic cell phosphatase, also referred to as SHP-1. The official gene symbols for the *me* and me^v mutations are $Hcph^{me}$ and $Hcph^{me\text{-}v}$, respectively.[49–51] These mutations cause severe hematopoietic dysregulation, a severe immunodeficiency, and systemic autoimmune disease. The mutant mice exhibit hematopoietic abnormalities characterized by impaired T and conventional B (B-2) lymphopoiesis, defects in natural killer cell function and differentiation, increased myelomonocytopoiesis and CD5 B (B-1) lymphopoiesis, and macrophage accumulation.[25,52] Along with increased B-1 cell lymphopoiesis and plasmocytic differentiation, macrophages in *me/me* and me^v/me^v mice show increased proliferative responses to GM-CSF, suggesting that GM-CSF is a major factor for macrophage development in mutant mice.[51] In me^v/me^v mice, we found a twofold increase in the GM-CSF level of peritoneal fluid, suggesting that GM-CSF can induce the development and differentiation of CD5$^+$ macrophages in the peritoneal cavity. To clarify the effects of GM-CSF on CD5$^+$ macrophage development in mice other than me^v/me^v mice, we injected GM-CSF (5 ng/day) subcutaneously into BALB/c, C3H/He, or *op/op* mice for 5 days. As a result, we confirmed that CD5$^+$ macrophages emerged and occupied approximately 20% of total peritoneal cells in the GM-CSF-treated normal mice, indicating that GM-CSF induces the development of CD5$^+$ macrophages. In contrast, compared with those of GM-CSF-treated wild-type me^v or *op* littermates (+/+) and normal mice, the percentages of CD5$^+$ peritoneal macrophages in GM-CSF-treated *op/op* mice were low (less than 2%). This result suggests that, besides GM-CSF, M-CSF is also required for CD5$^+$ macrophage development and differentiation *in vivo* as shown above *in vitro*, as the *op/op* mice are defective in production of functional M-CSF protein.[40,43,44,53]

Milky spots in the omentum are leukocytic and lymphocytic collections containing progenitors for macrophages[54–60] or lymphoid cells[19,61,62] and supply a microenvironment for the differentiation of macrophages[59,60] and lymphocytes.[19] It has been postulated that the milky spots supply macrophages into the peritoneal cavity[54–61] and that macrophages differentiate in the milky spots and migrate into the peritoneal cavity.[52,53] A previous study using ultrastructural peroxidase cytochemical approaches demonstrated that promonocytes and monocytes are present in the milky spots[55] and that their differentiation into macrophages is supported by M-CSF produced *in situ*.[59,60] CD5$^+$ B (B-1a) cells develop in the omentum, particularly in the milky spots, migrate into the peritoneal cavity, and are self-replenishing.[17,19] In the present study, we observed increased numbers of CD5$^+$ macrophages in the milky spots of GM-CSF-treated BALB/c mice, suggesting that these macrophages develop therein. As for the developmental process of CD5$^+$ macrophages, two possibilities are presented on the basis of the previous *in vitro* studies; one is their differentiation from a common precursor cell for CD5 B cells and macrophages[11,12,15,20,21,23,25] and the other a macrophage lineage switch of CD5 B cells.[14,22] In our current *in vitro* study, CD5$^+$ or B220$^+$ macrophages or CD5$^+$ and ER-MP20$^+$ or ER-MP58$^+$ macrophage precursor cells were demonstrated in the co-cultures of an established pre-B cell line, J13, and of fetal mouse liver cells, adult mouse bone marrow cells, or peritoneal wash cells with ST2 and GM-CSF. As ER-MP20 recognizes epitopes of colony-forming unit-macrophage, monoblasts, promonocytes, and monocytes and as ER-MP58 is a marker for myeloid precursor cells (M-CSF-responsive colony-forming unit-granulocyte/macrophage), the demonstration of CD5$^+$ ER-MP20$^+$ or ER-MP58$^+$ cells *in vitro* implies that there are common progenitor cells for CD5$^+$ macrophages and CD5 B cells.

To provide evidence for the presence of common progenitor cells for CD5$^+$ macrophages and CD5 B cells more definitely, we examined the changes in percentage of CD5$^+$ hematopoietic progenitor cells, CD5$^+$ macrophages, and CD5 B cells in the peritoneal cavity of BALB/c mice before and after daily GM-CSF administration by flow cytometry using both CD5 and one of the monoclonal antibodies for hematopoietic stem cells, macrophage precursor cells, macrophages, and B cells. In this analysis, we used CD34 and c-kit for detecting hematopoietic stem cells, as CD34 is known to be a marker for the stem cells[35] and as c-kit recognizes a receptor for stem cell factor.[36,37] As a result, we confirmed small percentages of CD5$^+$ hematopoietic progenitor cells (CD5 and CD34 or c-kit double-positive cells), CD5$^+$ macrophage precursor cells (CD5 and ER-MP58 or ER-MP20 double-positive cells), and CD5 B cells (CD5 and B220 or IgM double-positive cells) in the peritoneal cavity of untreated normal mice. However, CD5$^+$ macrophages were not detected. In contrast, numerous CD5$^+$ macrophages developed in the peritoneal cavity of GM-CSF-treated mice, together with increased percentages of CD5$^+$ macrophage precursor cells, CD5$^+$ hematopoietic progenitor cells, and CD5 B cells ($P < 0.01$). These data indicate that CD5$^+$ macrophages differentiate from CD5$^+$ hematopoietic stem cells via CD5$^+$ macrophage precursor cells. If CD5$^+$ macrophages are derived from mature CD5 B cells by a macrophage lineage switch of CD5 B cells, CD5 B cells must be reduced during the CD5$^+$ macrophage development and differentiation. On the contrary, our present investigation revealed that the percentage of CD5 B cells was,

rather, increased after GM-CSF injection, together with the CD5$^+$ macrophage development. These results suggest that GM-CSF administration up-regulates hematopoietic progenitor cells and induces their bidirectional differentiation into CD5$^+$ macrophages and CD5 B cells. In previous studies, it was reported that, in the co-cultures with ST2, differentiation of CD5 pre-B cell lines into CD5$^+$ macrophages and CD5 B cells was induced by GM-CSF and IL-3/IL-5, respectively.[20,63] It is known that GM-CSF, IL-3, and partly IL-5 have overlapping functions and that the receptors for GM-CSF, IL-3, and IL-5 share a common β-chain for signal transduction of these growth factors.[63–68] Thus, it may be speculated that GM-CSF induces the development and differentiation of not only CD5$^+$ macrophages but also CD5 B cells in the peritoneal cavity of GM-CSF-treated mice *in vivo*. Furthermore, our present immunohistochemical investigation demonstrated increases of hematopoietic stem cells and CD5$^+$ macrophage precursor cells and their differentiation into CD5$^+$ macrophages in the milky spots of omentum in the GM-CSF-treated mice.

In conclusion, GM-CSF in combination with M-CSF can induce a CD5$^+$ macrophage population in the peritoneal cavity; however, M-CSF is not essential but required. This population is thought to differentiate from a bipotential precursor for CD5 B cells and macrophages in the milky spots of omentum and in the peritoneal cavity, providing more definite evidence for a close developmental relationship between B cells and macrophages not only *in vitro* but also *in vivo*.

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