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The kinetics and distribution of different macrophage populations in the developing rat skin

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Summary. Macrophages play important roles in host defense and homeostasis. In contrast to adulthood, far less is known about macrophage populations in fetuses and neonates. Macrophages were evaluated in the developing rat skin at different anatomical sites (head, anterior dorsal, posterior dorsal, and abdomen) of F344 rats obtained on gestational days 18 and 20, on neonatal days 1-21, and at adult weeks 5-15. The numbers of macrophages in the epidermis, dermis or perifollicular areas that were positive for ED1 (exudative macrophages with activated phagocytosis), ED2 (resident macrophages), and OX6 (antigen-presenting cells) were evaluated. There were no differences in macrophage numbers among the anatomical sites. In the epidermis, only OX6 cells were seen, with gradually increased numbers in neonates and adults. In the dermis, many ED1 cells were already seen in fetuses, and the number peaked on neonatal day 4, and remained at that level until adulthood. By contrast, ED2 and OX6 cells began to be seen after birth and their numbers continued to increase until adulthood; ED2 cells were distributed diffusely in the dermis, whereas ED1 and OX6 cells were present exclusively in the upper dermis. In perifollicular areas, ED1, ED2 and OX6 cells began to be seen after birth, and their numbers gradually increased until adulthood. Some macrophages in dermal and perifollicular areas gave double-positive reactions to $ED1^+ED2^+$, $ED1^+OX6^+$ or $OX6^+ED2^+$. Increased mRNA levels of colony stimulating factor-1 and monocyte chemoattractant protein-1 appeared to correspond to the emergence of rat macrophages. Skin macrophages were shown to be heterogeneous in distribution and function; the information from this study should be very useful for future investigations of experimentally induced rat skin lesions.

Key words: CSF-1, Developing rat skin, Immunohistochemistry, Macrophage populations, MCP-1

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Introduction

Because it serves as the layer of tissue that comes in contact with surrounding environments, the skin of mammalian bodies has important roles in the host defense mechanism. Macrophages reside within normal human and mouse dermis as a major cellular element (Dupasquier et al., 2004; Ochoa et al., 2008), and dermal macrophages are important to the immunity and homeostasis of human skin (Ochoa et al., 2008). Macrophages have functions such as phagocytosis, antigen presentation, killing of pathogens, and production of inflammatory factors. They are not homogenous cell populations in location, morphology, function, or immunophenotype (Naito, 1993; Naito et al., 1996; Hume et al., 2002). Generally, macrophages are divided into exudative macrophages, resident macrophages (histiocytes), and antigen-presenting dendritic cells (Naito, 1993; MacDonald et al., 2005; Polfliet et al., 2006). These cells have been found in divergent sites of normal skin, and some are preferentially located in certain skin regions, e.g. Langerhans cells (LCs) in the epidermis (Eichmüller et al., 1998). Although distributions of skin macrophages in adult humans and mice have been reported, far less is known about their populations in fetuses and neonates. In rats, there are no sufficient data concerning the development, localization, and kinetics of skin macrophages from the gestational to adult period; such information is essential for the pathological evaluation of skin diseases.

To analyze macrophage heterogeneity, antibodies against surface and cytoplasmic molecules specific for rat macrophages have been produced. We used a panel of antibodies, namely ED1 (rat CD68, for exudative macrophages), ED2 (rat CD163, for resident macrophages), and OX6 (rat Ia antigen MHC class II, for antigen-presenting cells). These antibodies have been widely used to investigate macrophage functions in chemically induced rat renal and hepatic fibrosis (Ide et al., 2003; Yamate et al., 2004). In addition to the immunophenotypical analyses, mRNA expressions of macrophage colony-stimulating factor-1 (CSF-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage chemoattractant protein (MCP-1) were investigated in developing rat skin tissues. It has been reported that the generation, proliferation and differentiation of macrophages requires these growth factors or chemoattractants (Naito et al., 1997; Shibata et al., 2001).

Materials and methods

Animals

F344/DuCrj rats at the age of 4 and 9 weeks, as well as pregnant rats were obtained from (Charles River Japan, Hino, Shiga, Japan). These animals were housed in an animal room at a controlled temperature of 21±3ºC and with a 12-h light-dark cycle; they were provided a standard diet for rats (MF; Oriental Yeast Co, Ltd, Tokyo, Japan) and tap water *ad libitum*. Adult skin was prepared from rats at weeks 5, 10 and 15. Rat fetuses were taken from the pregnant rats on gestational days 18 and 20. After delivery, skin tissues were obtained from neonates on days 1, 4, 8, 15, and 21. At each examination point, two to four rats were euthanized by exsanguinations under ether anesthesia. Animal housing and sampling conformed to the institutional guidelines of animal care of Osaka Prefecture University.

Tissue preparation

At necropsy, skin tissues were collected from four different anatomical sites (head, anterior dorsal, posterior dorsal, and abdomen). The skin was immersed in periodate lysine paraformaldehyde (PLP) fixative for 4 hours at 4ºC, and then soaked in phosphate-buffered saline (PBS) at 4ºC for 2 hours. Tissues were embedded in paraffin by AMeX (acetone-methyl benzoate-xylene) methods (Suzuki et al., 2000). Deparaffinized sections were stained with hematoxylin and eosin (HE).

Immunohistochemical staining

Immunohistochemistry was performed using the Histofine® Simple Stain Mouse MAX PO method (Nichirei, Tokyo, Japan) with monoclonal antibodies. Primary antibodies used were ED1 (Chemicon International, Temecula, CA, USA), ED2 (AbD Serotec, Oxford, UK) and OX6 (AbD Serotec, Oxford, UK). To quench endogenous peroxidase, deparaffinized sections were treated with 3% H_2O_2 for 10 minutes; then, antigen retrieval was performed with 10% protease K (Dako Corp., Carpinteria, CA, USA) for 10 minutes. Skin sections were immersed in 5% skimmed milk for 30 minutes for protein blocking. Primary antibodies were applied at an appropriate dilution (ED1, 1:500; ED2, 1:400; OX6, 1:200) and incubated overnight at 4ºC. After being visualized using a 3,3'-diaminobenzidine (DAB, Vector Laboratories Inc., CA, USA), sections were lightly counterstained with hematoxylin. Sections used as negative controls were incubated with non-immunized mouse sera, instead of the primary antibody.

Double immunohistochemical staining

Co-expressions for $ED1^+ED2^+$, $ED2^+OX6^+$, and $ED1^+OX6^+$ were investigated with the double-immunolabeling method using a combination of horseradish peroxidase and alkaline phosphatase (HRP-AP). In the first staining, DAB was used as chromogen to yield a brown color, as described above. According to previously described methods (Hasui et al., 2003), the sections were then treated with 0.1M buffer glycine hydrochloride (pH 2.2) for 60 minutes to remove the residual primary antibody. The sections were incubated with the second monoclonal antibody for 14-24 h at 4ºC; the sections then were treated for 30 minutes with Histofine® simple stain AP solution (Nichirei, Tokyo, Japan), which includes goat anti-mouse Ig Fab' conjugated to alkaline phosphatase-labeled amino-acid polymers. Immunoreaction for the second monoclonal antibody was visualized red by fuchsin substrate-chromogen system (Dako Corp., Carpinteria, CA, USA) for 10 minutes. ED1 or OX6 was applied to the ED2-immunostained sections, and ED1 for the OX6-immunostained sections.

RNA extraction and real-time quantitative polymerase chain reaction (PCR)

Dorsal skin obtained at appropriate examination points was kept in RNA stabilization reagent RNAlater (Qiagen, Germany). Total RNA was isolated from the skin samples using SV Total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Afterwards, 0.5 μg of total RNA was transcribed with Superscript II reverse transcriptase using random hexamers (Invitrogen, Carlsbad, CA, USA). To determine relative expression levels of GM-CSF, MCP-1, and CSF-1 genes, SYBR green-based real-time PCR was performed using the LineGene system (BioFlux, Tokyo, Japan). The cycling conditions were as follows: 1 cycle of 95°C for 1 min, followed by 40 cycles of 95°C for 15 s, 62°C for 15 s, and 72°C for 20 s. The expression ratio relative to ß-

actin was calculated based on the threshold cycle value (Ct). Specific primers used were as follows: for GM-CSF (accession number XM0010 74265), sense primer 5'-CTGGAGAACGAAAAGAA CGAAGA-3' and antisense primer 5'-AGTCAGTTT CCGGGGTTGG-3'; for MCP-1 (accession number NM031530), sense primer 5'-GCTTCTGGGCCTGTTG TTC-3' and antisense primer 5'-CTGCTGCTGGTGA TTCTCTTGT-3'; for CSF-1 (accession number NM023981), sense primer 5'-ACAGGTGGAACTG CCGCCAGTGTAGAA-3' and antisense primer 5'-GGTGGACGTTGCCATAATGTCTC-3'; and for ß-actin (accession number BC063166) sense primer 5´-TAAAGACCTCTATGCCAACAC-3' and antisense primer 5-CTCCTGCTTGCTGATCCACAT-3'.

Cell counts

The numbers of cells reacting to ED1, ED2, OX6 were evaluated in three different sites, namely epidermis, dermis, and around hair follicles (perifollicular areas). Epidermal positive cells were determined by counting in a length of 0.5 mm of 5 different epidermis of each anatomical site. Cells showing distinct positive reactions in the dermis (papillary and reticular dermis) were quantified in 5 randomly selected fields (0.2 mm^2/field). For quantitative analysis in perifollicular areas, immunopositive cells were counted in 30 randomly selected hair follicles. The percentage of double-labeled cells per 0.2 mm^2 of the dermis was also calculated in five sections of each specimen (n=3).

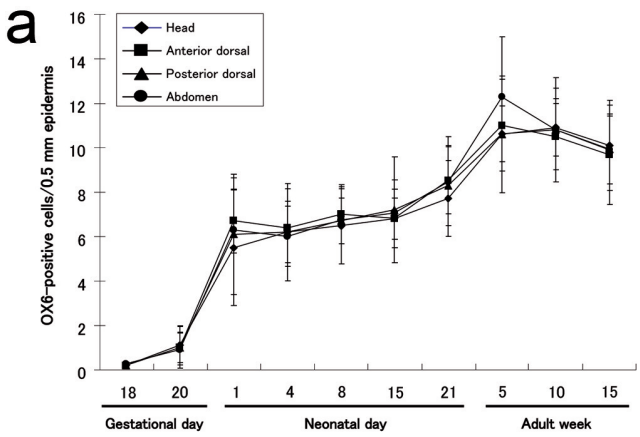
Statistical analysis

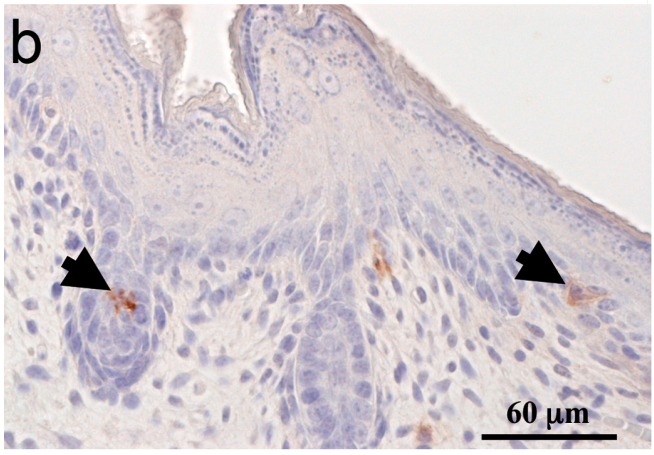
Samples were compared by Student's t-test or Tukey's multiple comparison test. Values of $P<0.05$ were considered statistically significant.

Results

Histology of the developing skin

On gestational day (GD) 18 the epidermal layer appeared as epithelia with 3-5 layers; on GD 20, this layer had developed into cornified, multilayered epithelia with clear squamous stratification. After birth, the structures of the squamous epithelial layer became more prominent with age. On GDs 18 and 20, hair germs were scattered throughout the developing dermis; the hair germs were characterized by a convex proximal end which was enveloped by the hair bulb and surrounded with mesenchymal cells. After birth, hair follicles underwent the cyclic process consisting of the anagen (growth) phase, catagen (regression) phase, and telogen (resting) phase. These hair follicles in the developing skin showed different stages as follows, depending on age (Müller-Röver et al., 2001): follicular neogenesis/early anagen on neonatal days (NDs) 1 to 15,





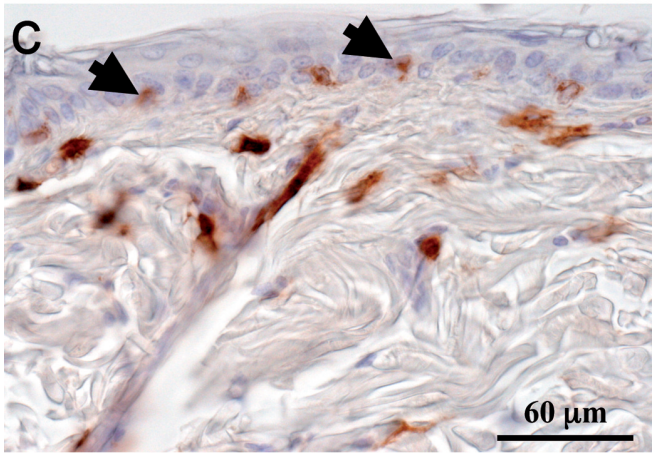


Fig. 1. The kinetics of OX6 cells (for antigen-presenting cells) in the epidermis. A few OX6 cells are seen in fetuses on gestational days (GDs) 18 and 20, and their number increases on neonate days (NDs) 1-21 and at adult weeks (AWs) 5-15; no difference is present between anatomical locations examined **(a)**. OX6 cells (arrows) are seen in the germ cell layer on GD 18 **(b)** and at AW 10 **(c)**. **b and c**, immunohistochemistry, counterstained with hematoxylin.

1st catagen on ND 21, full anagen at adult week (AW) 5, 2nd catagen at AW 10, and telogen at AW 15. At the catagen phase, apoptotic bodies were observed by *in situ* end-labeling using the terminal deoxyribonucleotide transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method (data not shown). In the epidermis, some apoptotic bodies were also confirmed by the TUNEL method.

Macrophage populations in the developing epidermis

In the developing epidermis, neither ED1- nor ED2-positive cells (ED1 and ED2 cells, respectively) were observed. A few OX6-positive cells (OX6 cells) were seen on GDs 18 and 20, and the number gradually increased on NDs 1 to 21 and AWs 5-15; the increased number reached a plateau at AW 5 (Fig. 1a). At each examination point, there was no significant difference in OX6 cell number between anatomic sites (Fig. 1a). OX6 cells were generally located in the basal part of the epidermis (Fig. 1b,c). OX6 cells in fetuses were round in shape with a large nucleus, whereas those in neonates and adults had a polygonal shape with dendritic processes (Fig. 1b,c).

Dermal macrophage populations in developing skin

Many ED1 cells were seen as early as fetuses on GD 18, and the number reached an adult-equivalent density since ND 4 (Fig. 2a). ED2 cells were rarely seen on GDs 18 and 20; however, after birth the number gradually increased in neonates and adults with a maximum number at AW 15 (Fig. 2b). Although the OX6 cell number on GDs 18 and 20 was very small, after birth it gradually increased until AW 15 (Fig. 2c). In terms of the numbers of ED1, ED2 and OX6 cells, there was no significant difference between anatomical sites examined (Fig. 2a-c).

As shown in Fig. 2d, among dorsal ED1, ED2 and OX6 cells, the ED1 cell number was the greatest on GD 20 and NDs 4 and 21. On GD 20, OX6 cells were the second most prevalent; on NDs 4 and 21, ED2 cells were second. At AW 10, the ED2 cell number overtook that of ED1 cells.

A few ED1 cells were evenly distributed throughout the dermal tissue on GDs 18 and 20 (Fig. 3a); in neonates and adults the cells tended be seen in the upper dermis, particularly underneath the epidermis, lining the dermo-epidermal junction (Fig. 3b); more interestingly,

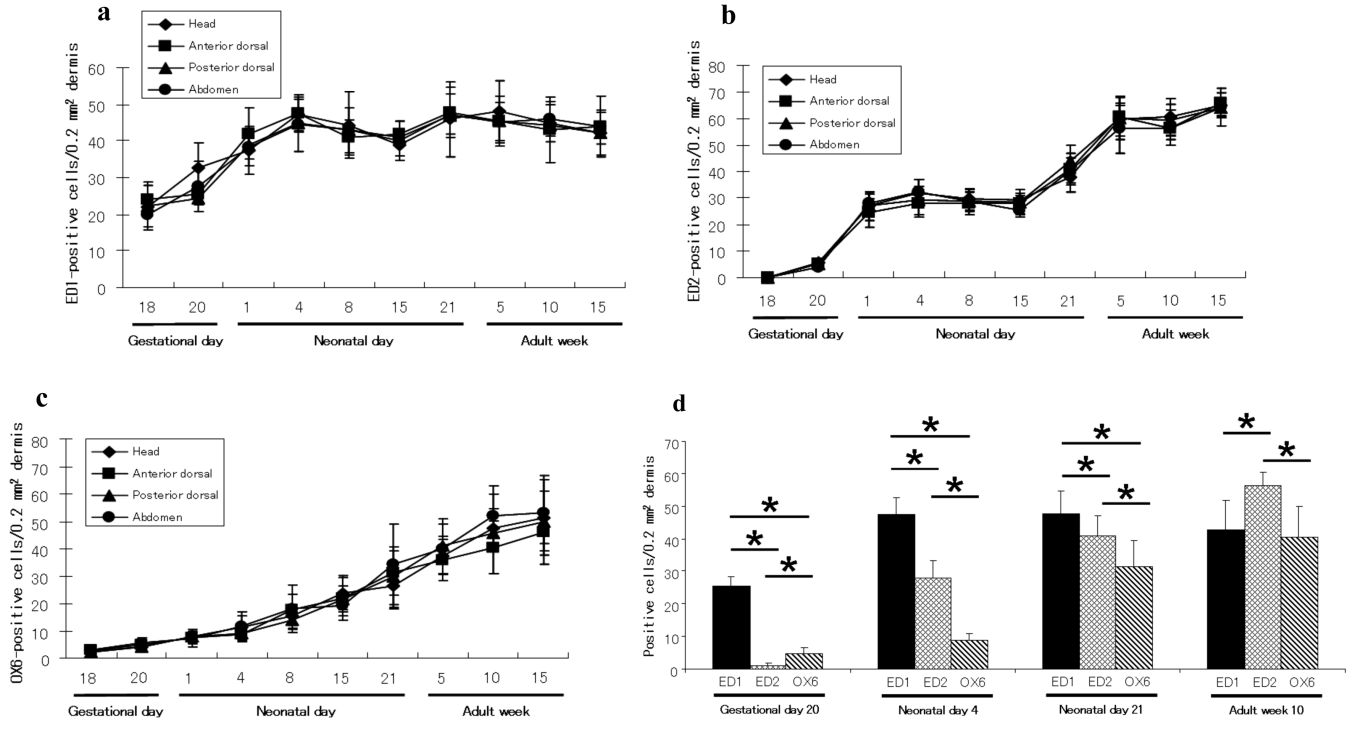


Fig. 2. The kinetics of cells labeled with ED1 (exudative macrophages with phagocytic activity) **(a)**, ED2 (for resident macrophages) **(b),** and OX6 **(c)** in the dermis, and the difference between macrophage populations at selected examination points **(d)**. There is no difference between anatomical locations examined **(a, b, c)**. ED1 cells are observed in fetuses, and on ND 4 the number peaks with adult-equivalent density **(a)**; on the other hand, ED2 and OX6 cells begin to be seen after birth, and the number continues to increase up to AW 15 (**b, c**). Among macrophage populations in dorsal skin (**d**), ED1 cells are most frequent in fetuses on GD 20 and NDs 4 and 21, whereas ED2 cells are greatest at AW 10. *: significantly different at $P<0.05$.

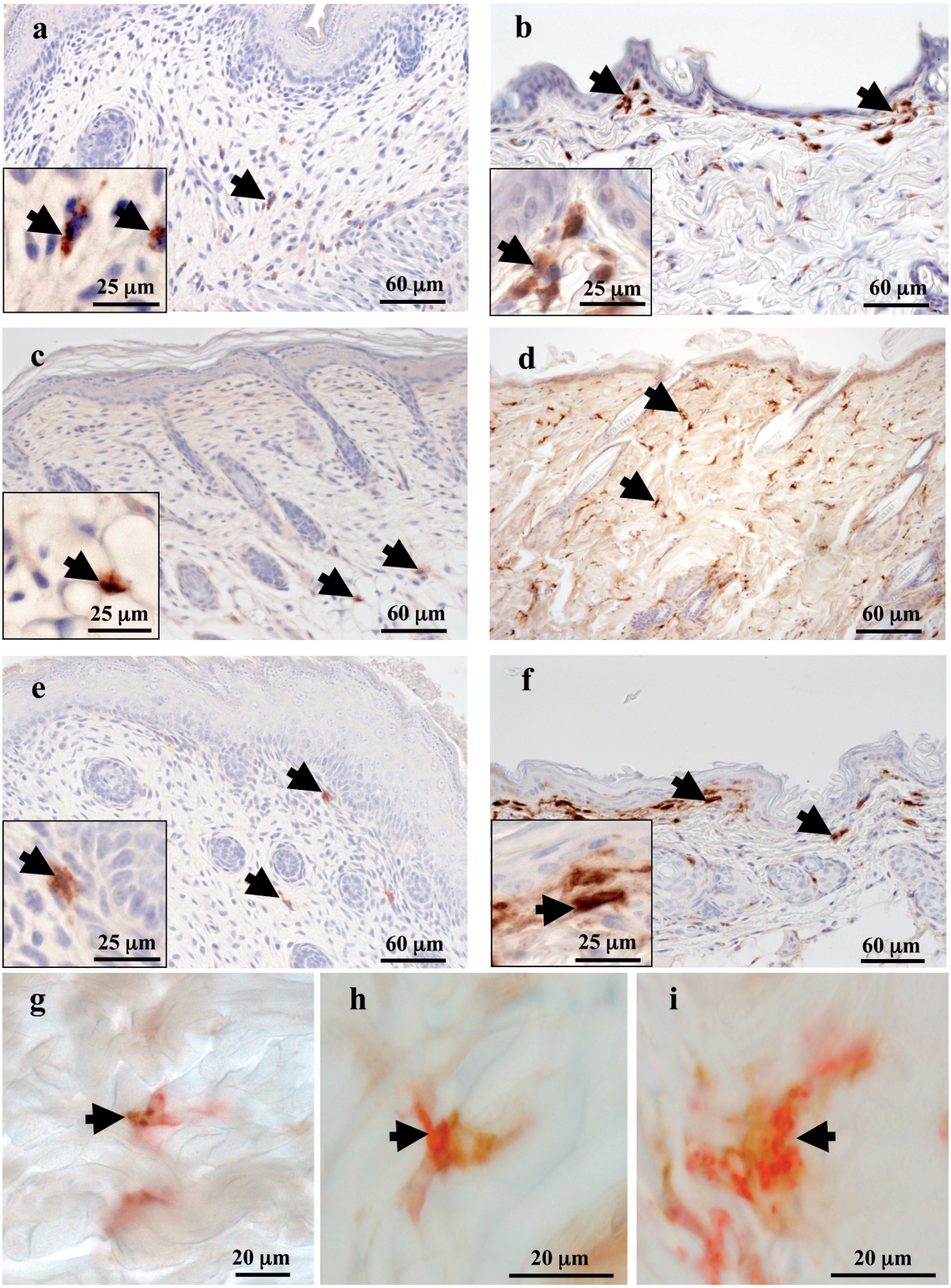


Fig. 3. Immunohistochemistry for cells in the dermis reacting to ED1 (**a, b**), ED2 (**c, d**), and OX6 (**e, f**). A few ED1-positive cells (arrow) are seen in the dermis on GD 20 (**a**), whereas many ED1-positive cells (arrows) are present under the epidermis (within papillary bodies) at AW 10 (**b**); **inset in a and b**, higher magnification of respective ED1 cells (arrows). ED2 cells (arrows) are sporadically seen on ND 4 (**c**), whereas many ED2 cells (arrows) are diffusely distributed among connective tissues in the epidermis at AW 10 (**d**); inset in **c**, higher magnification of ED2 cells (arrow). A small number of OX6 cells (arrows) are seen in the dermis on GD 20 (**e**), and increased number of OX6 cells (arrows) are localized exclusively under the epidermis at AW 10 (**f**); **inset in e and f**, higher magnification of respective OX6 cells (arrows). Double immunostaining (arrows) at AW 10, for ED1 (red) and ED2 (brown) (**g**), for ED2 (red) and OX6 (brown) (**h**), and for ED2 (red) and OX6 (brown) (**i**); ED2+OX6+ (**h**) and ED1+OX6+ (**i**) cells show dendritic appearance. a - f, single immunohistochemistry, counterstained with hematoxylin. g - i, double immunohistochemistry, not counterstained with hematoxylin.

aggregates of ED1 cells occasionally appeared in the papillary bodies (Fig. 3b). Although ED1 cells in adults were somewhat larger than those in fetuses and neonates, they had granular cytoplasm with a round nucleus (Fig. 3a,b). ED2 cells were round or dendritic in appearance, and were evenly distributed among connective tissues throughout the lower and upper dermis (Fig. 3c,d). OX6 cells were small and round without evident cell projections, pleiomorphic, having a bipolar structure, or dendriform, projecting several long cytoplasmic processes (Fig. 3e,f). Similar to ED1 cells, OX6 cells were predominantly located in the upper dermis, mainly under the dermis (Fig. 3f). There seemed to be a close relationship in the localization between epidermal and dermal OX6 cells (Fig. 1c).

Double immunohistochemistry using tissue samples from ND 4 and AW 10 revealed that there were cells reacting to $ED2^+ED1^+$ (Fig. 3g), $ED2^+OX6^+$ (Fig. 3h), or $ED1^+OX6^+$ (Fig. 3i). The percentages of these double-labeled cells are shown in Table 1.

Macrophage populations in the perifollicular region of developing skin

On GDs 18 and 20, hair follicles were not completely developed in the developing skin. After birth, ED1, ED2 and OX6 cells began to be seen in the vicinity of distal hair follicles, and their numbers were gradually increased up to AW 5 during the second

Table 1. The percentage (%) of double-labeled cells in developing rat skin.

Location	Percentage (%)	
	Neonatal day 4	Adult week 10
Dermis		
$ED2^+ED1^+$ per total $ED2^+$ cells	26.7	19.6
$ED2^+OX6^+$ per total $ED2^+$ cells	12.6	22.4
$ED1^+OX6^+$ per total $ED1^+$ cells	29.3	13.4
Perifollicular area		
$ED2^+ED1^+$ per total $ED2^+$ cells	< 10	< 10
$ED2^+OX6^+$ per total $ED2^+$ cells	< 10	< 10
$ED1^+OX6^+$ per total $ED1^+$ cells	< 10	< 10

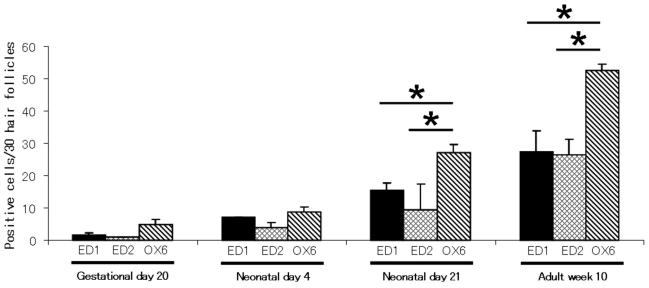


Fig. 4. The number of cells reacting to ED1, ED2, and OX6 in the perifollicular areas at selected examination points. OX6 cells are the most frequent on ND 21 and at AD 10, as compared with ED1 and ED2 cells. *: significantly different at P<0.05.

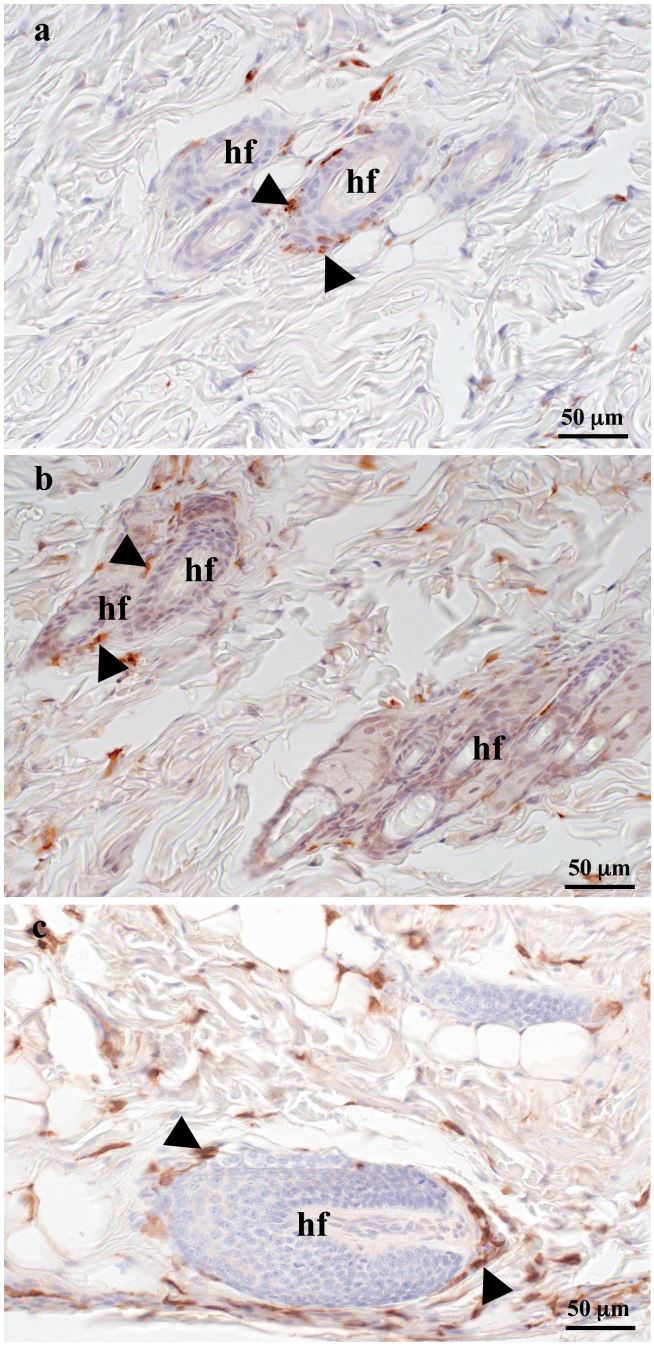


Fig. 5. The immunohistochemistry of cells reacting to ED1 (**a**), ED2 (**b**), and OX6 (**c**) at AW 10 in the perifollicular area. Hair follicles in the early catagen phase (hf, cross section of hair follicles) are clearly surrounded by macrophages (arrowheads) reacting to ED1 (a), ED2 (b) and OX6 (c). a - c, immunohistochemistry, counterstained with hematoxylin.

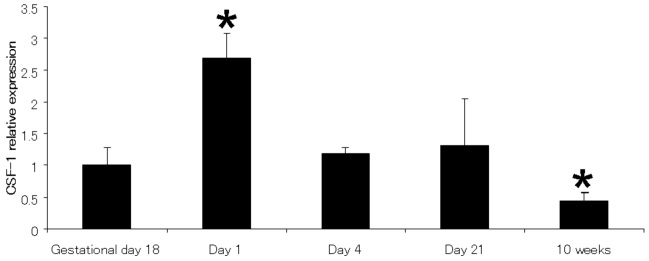


Fig. 6. mRNA expressions of colony stimulating factor-1 (CSF-1) in the developing skin tissues on GD 18, on NDs 1, 4 and 21, and at AW 10. The expressions are presented as relative levels, normalized to that of ß-actin. Three different samples at each point were analyzed. *: significantly different from gestational day 18 at P<0.05.

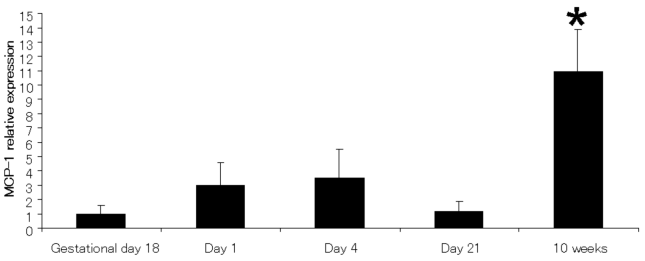


Fig. 7. mRNA expressions of monocyte chemoattractant protein-1 (MCP-1) in the developing skin tissues on GD 18, on NDs 1, 4 and 21, and at AW 10. The expressions are presented as relative levels, normalized to that of ß-actin. Three different samples at each point were analyzed. *: significantly different from gestational day 18 at P<0.05.

anagen of hair follicles. There was no significant difference between anatomical sites examined. As shown in Fig. 4, on ND 21 and at AW 10, the most frequently appearing cell was OX6 cells (Fig. 4). In the perifollicular area, the percentage of double-immunopositive cells was less than 10% as shown in Table 1. ED1, ED2 and OX6 cells in the perifollicular area showed the same morphology as seen in the dermis (Fig. 5a-c).

Real-time RT-PCR analysis

Compared to that on GD 18, CSF-1 mRNA expression was significantly increased on ND 1, but significantly decreased at AW 10 (Fig. 6). mRNA expression of MCP-1 did not show a significant change on NDs 1, 4 and 21, but significantly increased at AW 10 (Fig. 7). No significant expression level of GM-CSF mRNA was seen at any examination point.

Discussion

The present study showed that macrophages reacting to ED1, ED2 or OX6 appeared in developing rat skin tissues, indicating an important cell component in the skin. Macrophages play central roles for both the innate and acquired cutaneous immune system (Dupasquier et al., 2004; Stratis et al., 2006; Ochoa et al., 2008). It has been reported that there may be preferential anatomical sites for some autoimmune diseases, particularly for antigen-presenting cells (Banchereau and Steinman, 1998; Kanzler et al., 2007). However, no significant differences were found in the appearance of these macrophages between anatomical sites examined. On the other hand, there were differences in macrophage components between the histological sites evaluated (epidermis, dermis and perifollicular areas).

In the epidermis, only OX6 cells were present. OX6 recognizes MHC class II (rat Ia), and its expression implies antigen-presentation capacity (Kaneko et al., 2008). LCs are professional antigen-presenting cells present in the epidermis (Parent et al., 1989; Hussein, 2008). OX6 cells began to appear after birth on ND 1, and their numbers gradually increased until AW 5. In agreement with previous reports in adult human or mouse skin (Danielyan et al., 2007), the OX6 cells were located mainly in the germ cell layer of the epidermis. Likely, the epidermal OX6 cells correspond to so-called LCs, and the appearance might be induced by external environmental conditions (such as microorganisms and physical stimuli) after birth.

On the other hand, in the dermis, macrophages reacting to ED1, ED2 and OX6 were clearly observed. However, the kinetics and distribution patterns differed from each other. Many ED1 cells were seen as early as GD 18, and the number reached a plateau on ND 4, remaining at that level until adulthood. While ED1 cells were the most predominant in fetuses and neonates (until ND 21), ED2 cells came to be greatest in adulthood. Additionally, ED1 cells were aggregated in the papillary bodies of neonates and adults, whereas ED2 cells were evenly distributed among connective tissues in the upper and lower dermis. Similar to epidermal OX6 cells, dermal OX6 cells began to be seen after birth and then gradually increased until AWs 5-15; the location of OX6 cells was mainly in the upper dermis close to the epidermis. The ED1 and OX6 cells present in such histological sites may play important roles in skin autoimmune diseases, because a mononuclear cell reaction was observed in the dermo-epidermal junction in a murine model of early cutaneous lupus erythematosus (Menke et al., 2008).

The antigen (rat CD68) recognized by ED1 is located on the lysosomal membrane, particularly that of phagolysosomes; therefore, the increased expression implies phagocytic activities (Damoiseaux et al., 1994). Rat fetal dermal macrophages have been shown to be characterized by developing lysosomal granules and micropinocytic vesicles (Takahashi et al., 1983); dermal macrophages reacting to ED1 in fetuses appeared to reflect these ultrastructural findings. Many ED1-positive macrophages were localized within the papillary dermis, partly suggesting their roles in dealing with external threats which can enter via the epidermis or blood

stream. The epidermal layer is completed through stratified squamous cell differentiation from germ cells and apoptosis of cells that have fulfilled their functions (Gandarillas et al., 1999). In fact, some apoptotic bodies were seen in the epidermal layer. ED1 cells in the upper dermis might also be related to the quick removal of such apoptotic bodies or cell debris.

ED2 is used to identify the resident macrophages (histiocytes and Kupffer cells) (Ide et al., 2003; Fabriek et al., 2005). Therefore, ED2 cells seen among connective tissues were regarded as dermal histiocytes. The ED2-recognizing antigen (CD163) functions as a scavenger receptor for hemoglobin-haptoglobin complexes (Polfliet et al., 2006). ED2 cells may also be related to phagocytosis, like ED1 cells. Corresponding to the kinetics of dermal ED2 cells found in the present study, it has been reported that ED2 cells appeared immediately after birth in the thymus (Vicente et al. 1995), intestine (Van Rees et al., 1988), and lungs (Farver and Kobzik, 1999). Because the samples examined in the present study were from normally developing rat skin, dermal ED2 cells may contribute to skin homeostasis after birth by producing some growth factors (Suzuki et al., 1998), because the engagement of CD163 may reflect the production of proinflammatory mediators (Polfliet et al., 2006). ED2 cells appeared in inflammatory lesions of rats (O'Brien et al., 1998; Chbinou and Frenette, 2004; Yamate et al., 2009).

LCs in the epidermis are developed from dermal-resident dendritic cells expressing MHC class II under steady-state conditions (Larregina et al., 2001) and blood monocytes in skin inflammation (Ginhoux et al., 2006). There was a close relationship in the distribution and kinetics of OX6 cells between the upper dermis and epidermis. The dermal OX6 cells might be a precursor of LCs. Dendritic cells play crucial roles in cell-mediated immunity by acting as antigen-presenting cells to naïve T lymphocytes (Banchereau and Steinman, 1998; Kaneko et al., 2008). The OX6 cells appear to be cells that are prepared for encounters with external antigens and facilitate the subsequent complicated immune processes (Khalili et al., 1997).

Although perifollicular macrophages have been described (Eichmüller et al., 1998), the detailed cellular components remained to be investigated. The present study demonstrated that ED1, ED2, and OX6 cells started to be seen after birth in the perifollicular area, and their numbers continued to gradually increase until adulthood. Interestingly, although the perifollicular ED1 cells were as abundant as in the dermis, OX6 cells in the perifollicular area outnumbered ED1 and ED2 cells in late neonates and adulthood, indicating that there are differences between dermal and perifollicular macrophages. As mentioned above, the expression of ED1 antigens means activated phagocytosis (Damoiseaux et al., 1994). Because apoptotic cells were seen in developing hair follicles, perifollicular ED1 cells might be related to active removal of such cells during the late anagen and catagen phases of the hair cycle (Eichmüller et al., 1998). Apoptotic cells are considered to be a potent stimulus to macrophage recruitment for resolution and regeneration processes (Messmer and Pfeilschifter, 2000). However, a number of ED1-positive cells appeared in the perifollicular area, even when apoptosis was absent in the early stages on NDs 4-15, indicating another mechanism for ED1 cell recruitment. Fibroblast growth factor-5 produced by ED2 cells may regulate the hair growth cycle, particularly during anagen VI and catagen phases (Suzuki et al., 1998). Like epidermal antigen-presenting cells, perifollicular OX6 cells may play roles in host defense immune mechanisms. It will be interesting to investigate the functions of these macrophages with regard to hair development or hair cycles.

In addition to dermal and perifollicular macrophages showing different immunophenotypes, this study revealed that there were macrophages co-expressing ED1/ED2, ED2/OX6 or ED1/OX6, with percentages from 12.6 to 29.3% in the dermis and less than 10% in the perifollicular areas. $ED1^{+}ED2^{+}$ cells were considered to be resident macrophages with activated phagocytosis (Miyauchi et al., 1998; Sato et al., 1998). ED1+OX6+ and $ED2^{+}OX6^{+}$ cells might be exudative or tissue macrophages with some antigen-presenting capacity; the functions may be related to driving proliferation of antigen-experienced CD4 T cells (Chong et al., 2004; Blander and Medzhitov, 2006). The expression mechanisms of dual-function of dermal and perifollicular macrophages remain to be investigated.

Differences in the local production of growth factors such as CSF-1, MCP-1, and GM-CSF may contribute to differential macrophage recruitment (Stanley et al., 1997; Shibata et al., 2001; Hume et al., 2002). Recently, it has been reported that the development of dendritic cells, particularly LCs, are dependent on locally produced CSF-1 (MacDonald et al., 2005; Ginhoux et al., 2006). A significantly increased mRNA level of CSF-1 was seen on ND 1 when OX6 cells in the epidermis, dermis and perifollicular areas and ED2 cells in the dermis and perifollicular areas began to be seen. It is likely that CSF-1 might influence the very early appearance of ED2 and OX6 cells. Because many ED1 cells were already seen in the dermis of fetuses and neonates, there was no close relationship between increased CSF-1 expression and ED1 cell appearance. MCP-1 is a well-known chemokine playing an important role in recruitment and maintenance of macrophages in inflammation (DiPietro et al., 2001). MCP-1 showed a significant increase in mRNA expressions at AW 10 when ED1, ED2 and OX6 cells had reached their maximum numbers. The increased expression of MCP-1 might have been related to the settlement of these macrophages. Alteration of GM-CSF mRNA was not detected in developing rat skins. The relationship of these growth factors with macrophage recruitment and functions in the developing skin should be investigated in future studies.

In conclusion, the present study showed differences

in the kinetics and distribution of macrophage populations in developing rat skins between the epidermis, dermis and perifollicular areas. These macrophages appear to be related to homeostasis, immunity and modeling of the skin. A variety of skin lesions have been reported in autoimmune diseases, malnutrition, visceral organ insufficiency, endocrinopathy or medical drug-related side effects (Stratis et al., 2006). In wound healing, macrophages have a pivotal role in the transition between inflammation and repair by producing tumor necrosis factor-α, transforming growth factor-ß, and platelet-derived growth factor (Singer and Clark., 1999). The kinetics and functions of skin macrophages depend on various factors, both known and unknown. In dermatopathology, it is essential to understand the kinetics and distribution of heterogeneous macrophages appearing in normally developing skin as baseline information for investigating their participation and functions in diseased skin.

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